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Effects of Inbreeding on Endangered Red Wolves (Canis rufus)

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EFFECTS OF INBREEDING ON ENDANGERED RED WOLVES (*Canis rufus*)

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Dedicated to the inspired and committed members of the Red Wolf Recovery Team who have kept red wolves roaming free since 1989. Especially Chris Lucash, who is a constant source of engaging conversation and true biological insight.

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ABSTRACT

Inbreeding depression, the reduction in offspring fitness caused by mating among close relatives, is widespread in small populations and a major concern in conservation biology because it can affect population persistence. The negative effects of inbreeding results in the evolution of inbreeding avoidance behaviors; within small populations, such behaviors may encourage individuals to select mates outside of their respective species. Mate choice may also be facilitated by variation at major histocompatibility complex (MHC) genes, a gene group critical for immune response and disease resistance. Given broad impacts of inbreeding and MHC variation on fitness and behavior, evaluating their effects is an important component of wildlife management. My dissertation research examined how inbreeding and immunogenetic variation influenced fitness, disease susceptibility, and mating behavior in endangered wild red wolves (*Canis rufus*). I also evaluated mitochondrial DNA from ancient canid bones to inform an ongoing debate regarding the species status of red wolves. I found evidence for an ancient red wolf origin which supports contemporary red wolf management practices (Chapter 2). Although these analyses were not directly related to inbreeding, clarifying red wolf taxonomic status is vital for effective species conservation.

With regard to inbreeding depression, I found that red wolves were extremely inbred but their fitness was not associated with inbreeding. However, more inbred wolves tended to be smaller, which may have an indirect effect on reproductive success (Chapter 3). Next, I evaluated how immunogenetic variation influenced disease susceptibility by collecting baseline disease prevalence in red wolves and sympatric coyotes (*Canis latrans*), and sequencing MHC and toll-like receptor (TLR) genes. Coyotes harbored more parasite species then wolves and may act as disease reservoirs for red wolves (Chapter 4). Red wolves had

lower immune gene variation then coyotes; variation may have been maintained through positive selection at MHC genes (Chapter 5). There were also several TLR haplotypes which were correlated with disease susceptibility. Finally, I evaluated red wolves' mate choice (Chapter 6). I found little evidence for pedigree kinship avoidance but red wolves may avoid mates with more similar MHC alleles. This could contribute to hybridization with coyotes to avoid MHC-similar mates.

CHAPTER 1: GENERAL INTRODUCTION

INBREEDING IN CONSERVATION BIOLOGY

Inbreeding is common in small wild populations and can directly affect population persistence by decreasing survival and reproductive success (Keller and Waller 2002). The deleterious effects of inbreeding, called inbreeding depression, are attributed to an increase in genome-wide homozygosity, which causes expression of deleterious recessive alleles (dominance hypothesis) and/or loss of heterozygous advantage (overdominance hypothesis (Charlesworth and Wallis 2009). Although the degree of inbreeding depression may vary among populations, theoretically, no species or population is invulnerable to inbreeding (Lacy 1997, Crnokrak and Roff 1999, O'Grady et al. 2006).

Due to fitness costs associated with inbreeding, some species may have evolved inbreeding avoidance behaviors, particularly social and cooperatively breeding species, which encounter relatives more often than non-cooperative species (Pusey and Wolf 1996, Jamieson et al. 2009). Inbreeding avoidance behavior in small, inbred populations with limited mating opportunities could cause individuals to hybridize with members of a closely related species. Mate choice and inbreeding avoidance may also be facilitated by variation at major histocompatibility complex (MHC) genes (Grob et al. 1998, Sommer 2005). The MHC is a highly variable gene complex which plays a critical role in cellular immune response. Correlations between MHC alleles, haplotypes, or heterozygosity and pathogen resistance have been shown for a number of species (reviewed in Sommer 2005). Because MHC variation so strongly affects disease resistance, individuals may select mates to produce heterozygous offspring or offspring with advantageous MHC alleles/ haplotypes (Landry et al. 2001).

In addition to influencing mating behavior, inbreeding can affect disease susceptibility because increased homozygosity can reduce a population's ability to cope with newly introduced or evolving parasites and pathogens (Spielman et al. 2004). For example, a bottlenecked island population of Artic fox (*Vulpes lagopus semenovi*) with lower genetic diversity than mainland populations suffered a population crash due to epizootic mange, which continues to be a limiting factor for fox populations today (Ploshnitsa et al. 2011). Given this, inbreeding in association with low MHC variation could reduce pathogen resistance and immunocompetence, the ability to mount an immune response, in wild populations and contribute to extinction events.

Understanding how inbreeding and MHC variation influence mate choice and disease susceptibility is an important line of inquiry given that disease is recognized as a global threat to biodiversity (de Castro and Bolker 2004). Disease can contribute to extinction, and because climate change and human introductions appear to be shifting the geographic range of diseases, wildlife must be able to contend with new pathogens (Allendorf et al. 2001, Lafferty 2009). Additionally, climate-driven range shifts and human transportation have caused species previously allopatric to come into contact, increasing levels of hybridization and the potential for genetic extinction through introgression (Lafferty 2009). Thus, understanding factors influencing disease susceptibility and hybridization in natural populations is of growing importance to conservation issues.

One species for which inbreeding and mate choice analyses are important is the red wolf (*Canis rufus*). Red wolves are one of the few wild species with a multi-generational pedigree and extensive fitness data, which provides the unique opportunity to answer theoretical questions about how inbreeding and MHC variation may affect parasite resistance and hybridization generally.

STUDY SYSTEM AND OBJECTIVES

Historically red wolves were abundant throughout the eastern and southeastern United States, but populations were decimated in the 20th century due to habitat loss, intense predator control programs, hybridization with coyotes (*Canis latrans*), and disease, and the species was declared extinct in the wild by 1980 (Phillips and Parker 1988; Hinton et al. 2013). In the 1970s, the last remaining red wolves were trapped in southwestern Louisiana and southeastern Texas to start a captive breeding program (Fig. 1.1). Over four hundred wild canids were trapped but only 17 were deemed 100% red wolf; of these 14 successfully bred to become the founders of all contemporary red wolves (Phillips et al. 2003). The first captive red wolf litter was born in 1977 (United States Fish & Wildlife Service 2014), and after a few generations of captive breeding, the United States Fish and Wildlife Service (USFWS) released captive born wolves onto barrier islands off the coast of South Carolina, Florida, and Mississippi to confirm captive breeding did not reduce red wolf hunting ability. Wolves successfully secured prey and mated independently, suggesting captive breeding did not reduce red wolves ability to survive in the wild, and reintroduction efforts were initiated on the mainland in the late 1980's.

Two populations of red wolves were reintroduced, one in northeastern North Carolina (1987) and one in the Great Smoky Mountains National Park, Tennessee (1991; Fig. 1.1). In 1998, Tennessee restoration efforts were discontinued due to poor pup survival associated with malnutrition and possibly parasites and CPV infections (Henry 1998). As a result, the northeastern North Carolina population represents the only wild red wolf population (United States Fish & Wildlife Service 2014). The wild red wolf population grew throughout the 2000s, however, the population has recently decreased (United States Fish & Wildlife Service

2014; Fig. 1.2). The decrease in population size may be due to anthropogenic mortality, which is currently the greatest threat to wild red wolves (Hinton et al. *in press*).

Figure 1.1. Range of the contemporary reintroduced red wolf (*Canis rufus*) population in northeastern North Carolina (orange), the failed reintroduction site at Great Smoky Mountain National Park, Tennessee (gray), and location were remnant wild red wolves were trapped in the late 19070s to create the red wolf captive breeding program (blue).

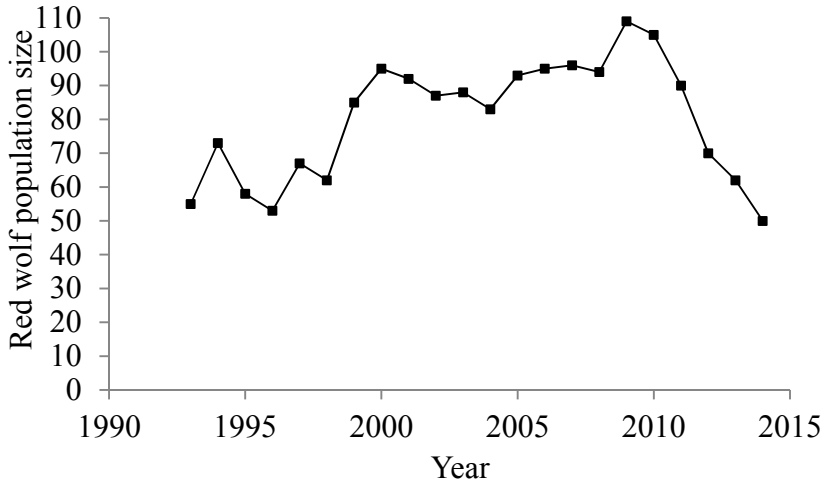

Figure 1.2. Population size of known wild red wolves (*Canis rufus*) in the recovery area in northeastern North Carolina.

The red wolf recovery area was coyote-free when wolves were reintroduced in the late 1980s, but due to coyote range expansion eastward, hybridization was observed starting in 1993. Hybridization was at that time considered the primary threat to reintroduced red wolves because genetic swamping due to coyote introgression could render red wolves functionally extinct (Fredrickson and Hedrick 2006). The threat of hybridization prompted the development of an adaptive management strategy to prevent further introgression of coyote genetic material into the wild red wolf population (Kelly et al. 1999; Stoskopf et al. 2005; Rabon et al. 2013). The adaptive plan included sterilizing coyote and hybrids to use as placeholders on the landscape to suppress coyote reproduction and prevent red wolves that paired with coyotes from producing hybrid litters (Hinton et al. 2013, Gese and Terletzky 2015, Hinton et al. in press). Ideally, sterile placeholders would naturally be displaced by red wolves. The placeholder strategy was largely successful at reducing introgression, where introgression and hybridization has been estimated to be below 4% in the wild red wolf population (Gese et al. 2015).

Complicating red wolf recovery is an ongoing red wolf species debate. There are several competing hypotheses of red wolves' taxonomic origin. Red wolves may have evolved as a distinct lineage in NorthAmerica from a coyote-like ancestor (Nowak 1992, 2002, Nowak et al. 1998, Chamber et al. 2012) and may be conspecific with eastern wolves, another possible wolf species endangered in the northeast (*C. lupus lycaon or C. lycaon*; Wilson et al. 2000, Kyle et al. 2006, Rutledge et al. 2012, Wilson et al. 2012, Rutledge et al. 2015). Alternatively, red wolves may represent a hybrid between gray wolves and coyotes (Wayne and Jenks 1991, Roy et al. 1994, 1996), possibly appearing only within the last 430 years (vonHoldt et al. 2011). If red wolves are a contemporary hybrid, conservation efforts would potentially be unwarranted. Historic and contemporary hybridization with coyotes,

overlapping ranges, and small population size have contributed to the taxonomic confusion of red wolves.

Understanding how factors like inbreeding influences fitness and hybridization can improve red wolf management and help answer broader questions of taxonomy. Given wild red wolves persist in a single small population, with few unrelated individuals available as mates, levels of inbreeding may be high and red wolves may hybridize with coyotes to avoid mating with kin. Red wolves may also select mates more dissimilar at important functional genes like MHC. The degree of inbreeding, inbreeding depression, inbreeding avoidance, and their effect on hybridization with coyotes is unknown for red wolves. An additional concern is whether inbreeding and MHC variation influences disease susceptibility; red wolf viability had already been critically affected by disease in the remnant Louisiana-Texas population and the Smoky Mountain site, and contemporary wild red wolves in North Carolina could be vulnerable as well. My dissertation attempts to answer these questions.

Although my dissertation focuses on broad impacts of inbreeding, I also analyzed mitochondrial DNA (mtDNA) from three ancient (350-1,900 year old) putative wolf samples excavated from middens and sinkholes within the historic red wolf range to clarify red wolf taxonomy. These results are presented in Chapter 2, and have been submitted for publication to Journal of Heredity. Chapter 3 evaluates inbreeding and inbreeding depression using pedigree inbreeding coefficients and long term fitness data; this manuscript was published in Molecular Ecology in 2014. My fourth chapter is a review of historic and potential contemporary disease threats to red wolves. I also collected baseline disease data and compared prevalence between red wolves and coyotes; these results were published in The Journal of Mammalogy in 2015. For my fifth chapter, I sequenced both innate, toll-like receptor (TLR) and adaptive major histocompatibility complex (MHC) immune genes, and

collected immunological and disease data from red wolves and sympatric coyotes to assess: 1) immunogenetic variation; 2) selection at immune genes; and 3) associations between immune genes and immune response or pathogen load. Finally, to better understand what influences hybridization, I assessed inbreeding avoidance and MHC-mediated mate choice in chapter 6. These last two chapters have not yet been submitted for publication.

Results from my dissertation can help red wolf recovery by informing managers about actions needed to increase reproductive success and survival, such as screening potential red wolf mates for genetic compatibility or increasing vaccination regimes to reduce pathogen infections. Additionally, my work broadly addresses fundamental processes by which individuals and populations persist, thereby supporting the conservation of biodiversity, an issue of substantial concern to diverse members of society.

Wild red wolf recovery has had many successes, such as being the first successful US program to remove a species from the wild to prevent extinction. Another achievement is the adaptive management plan to reduce hybridization; despite these successes, red wolf recovery faces real difficulties. Anthropogenic mortality may be disrupting breeding pairs, facilitating hybridization; habitat loss due to sea-level rise may substantially reduce the current red wolf recovery area; development and roads could fragment habitat to levels unsuitable for wolves; new disease spread by more abundant species like coyotes could affect red wolves; and the lack of political will to continue wolf conservation in the United States may all contribute to red wolves once more becoming extinct in the wild. The red wolf program successes are in part due to management practices based on the best available science; continued success will similarly rely on quality science, necessitating research like I present here.

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CHAPTER 2: MITOCHONDRIAL DNA VARIATION IN SOUTHEASTERN PRE-COLUMBIAN CANIDS

INTRODUCTION

The taxonomic status of North American eastern wolves has been debated over many years (Nowak 1979, 1992, 2002, Wayne and Jenks 1991, Roy et al. 1994, 1996, Nowak and Federoff 1998, Wayne et al. 1998, Wilson et al. 2000, Murry and Waits 2007, vonHoldt et al. 2011, Chambers et al. 2012, Rutledge 2015), yet this debate has yielded little consensus on species delimitations. One hypothesis proposes that the eastern United States was historically inhabited by a wolf-like canid that experienced serious population declines following human colonization from Europe (Goldman 1937, Wilson et al. 2000, Nowak 2002, Chambers et al. 2012) via anthropogenic habitat degradation and extermination programs, which also facilitated the spread of coyotes eastward (*Canis latrans*; Parker 1995). This resulted in extirpation and hybridization among various canid populations in the east (Wayne and Jenks 1991, Lehman et al. 1991, Hailer and Leonard 2008, Rutledge et al. 2010; Figure 1).

At the center of the eastern canid species debate is the endangered red wolf (*Canis rufus*), a putative southeastern wolf species that currently persists in one small, reintroduced population in North Carolina (Hinton et al. 2013). Red wolves may have evolved as a distinct lineage in North America from a coyote-like ancestor (Nowak 1992, 2002, Nowak et al. 1998, Chambers et al. 2012) and may be conspecific with eastern wolves (*C. lycaon* or *C. lupus lycaon*), another putative wolf species with debated nomenclature that is found primarily in Algonquin Provincial Park and adjacent areas in Ontario (Wilson et al. 2000, Kyle et al. 2006, Benson et al. 2012, Rutledge et al. 2012, Wilson et al. 2012, Rutledge et al. 2015).

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Alternatively, red wolves may represent a hybrid between gray wolves and coyotes (Wayne and Jenks 1991, Roy et al. 1994, 1996), possibly appearing only within the last 430 years, i.e., since the European invasion of North America (vonHoldt et al. 2011).

Historic and contemporary hybridization with coyotes, overlapping ranges, and small population size have contributed to the taxonomic confusion of red wolves (Wayne and Jenks 1991, Adams et al. 2003a, Wilson et al. 2003, Hailer and Leonard 2008). Yet identifying distinct lineages is important for implementation of the Endangered Species Act, which does not have a clear rule for the management of recent hybrids (Allendorf and Luikart 2007). Understanding evolutionary origins and historic distribution of eastern canids also is broadly important for wolf conservation in the United States. For instance, the conservation of Great Lakes wolves, a unique population of gray wolves in the Great Lakes region of the United States, Ontario, and Quebec, was jeopardized when gray wolves were delisted from the endangered species list in 2012. The 2012 delisting also removed protection for Great Lakes wolves until a 2014 federal court decision relisted them as a distinct population (USFWS 2014). The historic range and taxonomic status of Great Lakes wolves, which likely hybridized with eastern wolves and/or coyotes (Koblmüller et al. 2009, Wheeldon et al. 2010), was a critical aspect of the initial controversial delisting of gray wolves (Morell 2014, NCEAS 2014). Similarly, the Red Wolf Recovery Program recently underwent an intensive review in which the taxonomic status of the red wolf was once again questioned (Wildlife Management Institute 2014).

Examining the identity of canids found in the historic red wolf range prior to population declines and hybridization is critical to understanding how disturbance and biogeographic processes led to the contemporary canids now found in the southeastern United States (Rutledge et al. 2010). For example, if red wolves are the result of coyote-gray wolf

hybridization within the last 500 years, gray wolves would have needed to inhabit some portion of the southeastern United States during the pre-Columbian period. The paleontological record supports a wolf-like canid continuously inhabiting the southeastern United States since the terminal Pleistocene (Nowak 2002), but putative wolf samples from before European colonization have not been evaluated genetically. Given the paucity of data regarding the type of canid present prior to broad landscape changes, extirpation, and hybridization, I examined historic genetic samples from the southeastern United States. Specifically, I analyzed three canid DNA samples from the pre-Columbian period to assess the identity of the southeastern canid lineage.

METHODS

I analyzed three putative wolf teeth from within the historic red wolf distribution (Table 2.1, Figure 2.1), ranging in age from 350-1,900 years old. The teeth were considered late-Woodland period and were aged either based on faunal assemblages and early human activity (CM 038379) (Guilday et al. 1962, Guilday 1982), carbon dating of associated human remains (CM 0006548) (Jackson 1987), or stratification and early human activity (UMI 91100) (Futato and Solis 1983) at the their respective sites. Morphological analyses were previously conducted on all three samples, and they were identified as red wolve teeth based on their significantly different size than homologues in both coyotes and gray wolves (Table 1 in Nowak 2002).

Table 2.1. Accession number, museum, age (years before present; ybp), material sampled, state and county location, and local site for ancient DNA specimens sampled for mitochondrial DNA tests.

Accession number	Museum	Age (ybp)	Material sampled	Collection location	Archaeological Site
CM 038379	Carnegie Museum of Natural History	350	Tooth	Pennsylvania, Lancaster	Eschelman Site

(Table 2.1 continued)

Accession number	Museum	Age (ybp)	Material sampled	Collection location	Archaeological Site
UMI 91100	University of Michigan Museum	1,000	Tooth	Alabama, Jackson County	Crow Island Indian midden
CM 0006548	Carnegie Museum of Natural History	1,900	Tooth	Pennsylvania, Bedford County	New Paris Sinkhole No. 2

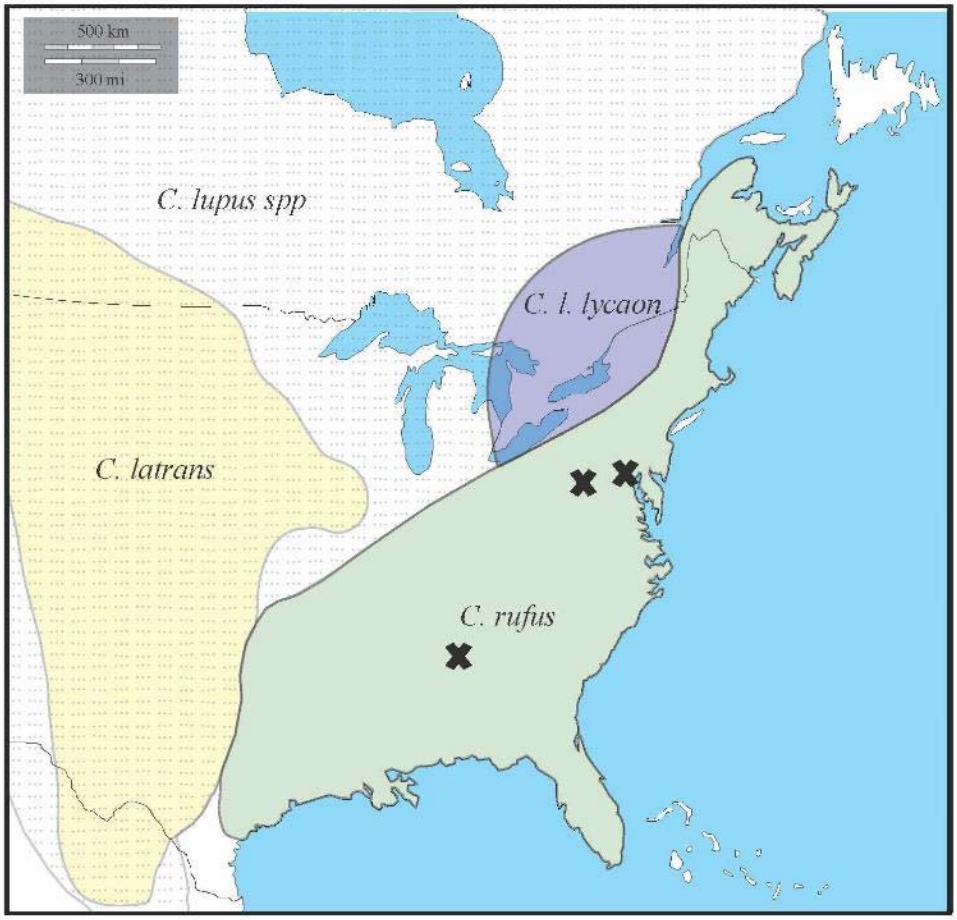

Figure 2.1. Historic map of North American *Canis* species and approximate sampling locations (Xs) for ancient DNA samples. Distributions are based on Parker 1995, Nowak 2002, and Chambers et al. 2012; for alternative range distributions see Kyle et al. (2006) and Rutledge et al. (2010).

I conducted all DNA extractions and polymerase chain reaction (PCR) set-up in a genetics lab dedicated exclusively to ancient DNA (aDNA) analyses. DNA was isolated from teeth following the column-based aDNA extraction method outlined in Rohland et al. (2010).

Prior to DNA extraction, I submerged all tooth samples in 6% bleach for 15 minutes to remove possible contaminants from the external surface (Kemp and Smith 2005), and manually ground samples to a fine powder with liquid nitrogen and a mortar and pestle cleaned with bleached and distilled water. I treated reagents and consumables following Champlot et al. (2010). I placed all tubes (clear-walled), PCR strips, water, rabbit serum albumin, and buffer within 1 cm of UV bulbs and irradiated them under UV light for 15 min. I treated dNTPS and Qiagen Hotstart *Taq* (Qiagen, Inc., Valencia, CA) with heat-labile double-strand specific DNase (Biotec Marine Biochemicals, Tromsø, Norway). I targeted the mitochondrial DNA (mtDNA) control region, previously found to have a unique red wolf haplotype (Adams 2003a), with four primer pairs that generated overlapping sequences. The resulting amplicons were concatenated to produce a 450 base pair sequence (Leonard et al. 2002; Table A1). I sent PCR product to Beckman Coulter Genomics (Danvers, MA) for bi-directional Sanger sequencing.

To ensure sequence reliability, I extracted DNA from every sample in two independent extractions and each DNA extract was amplified and sequenced at least four times with all four primer pairs (Table A2); I included several negative controls in every extraction and PCR to monitor contamination. I cloned and sequenced amplicons from two primer pairs for each putative wolf sample to detect DNA damage or potential contamination (Pääbo et al. 2004); PCR product was sent to MClab (San Francisco, CA) for cloning and sequence verification. Sequences were edited and compared with SEQUENCHER v5.0; replicate DNA extractions were treated as independent samples and then compared to create a final concatenated sequence for each individual. If there were ambiguous sites, I considered them resolved when two additional PCR reactions, overlapping sequences from flanking primer pairs, or cloning

confirmed a base. All three mtDNA sequences were deposited on Genbank (Accession numbers: *in progress*).

I aligned my aDNA sequences with mtDNA control region sequences previously published on GenBank using the MUSCLE algorithm (Edgar 2004) implemented in Geneious v8.1 (Kearse et al. 2012). Comparison sequences included likely potential species my samples could represent: domestic dogs, gray wolves, Great Lakes wolves, red wolves, and coyotes (Table A3). I used a red fox control region sequence (accession number AM181037) as the outgroup because its length reduced the number of nucleotides that I had to trim from the full alignment, as opposed to more closely related, but poorly overlapping Ethiopian wolf (*Canis simensis*) or Golden jackal (*Canis aureus*) sequences. I estimated the mtDNA control region gene tree using Bayesian and maximum likelihood (ML) methods from alignments including and excluding the outgroup sequence. In BEAST v1.8.2 (Drummond et al. 2012), I estimated a gene tree using the constant size coalescent tree prior and an uncorrelated lognormal relaxed molecular clock. I used a random starting tree, allowing the root of the tree to be one of the parameters that BEAST estimates. Two independent Markov chain Monte Carlo (MCMC) analyses were run for 25 million steps, sampling every 2500 steps. I determined convergence on the posterior distribution by viewing the log files in Tracer v1.6. Convergence on the posterior is attained when the effective sample size (ESS) of a parameter (i.e. the number of effectively independent draws from the posterior distribution) is at least 200. All parameters in my analyses had ESS values greater than 300. I combined tree files in LogCombiner v1.8.2 with a 10% burnin for each file and calculated the maximum clade credibility (MCC) tree for the combined tree file in TreeAnnotator v1.8.2. I estimated a ML tree, performed 1000 bootstrap replicates, and calculated the 50% majority rule consensus tree using the GARLI v2.1 web service (Bazinet et al. 2014).

RESULTS

All three ancient canid samples had unique mtDNA haplotypes not previously described and yielded composit sequences 450 basepairs in length. The validity of the aDNA sequences was supported by: 1) their similarity to modern canid haplotypes (no new indels or transversions); 2) no detected contamination in extraction, PCR, or cloning, and; 3) PCR and cloning replicates that were either identical or consistent across most sequences to resolve questionable sites (Table A2). There were, however, five ambiguous sites in CM 038379, three ambiguous sites in UMI 91100, and three ambiguous sites in CM 0006548 that I was unable to resolve through replicate PCR or cloning. Unresolved sites were all pyrimidine ambiguities (cytosine or thymine) suggesting there was some DNA damage caused by deamination of cytosine, a common issue with aDNA (Hofreiter et al. 2001). My three aDNA sequences were unique no matter which base was used at ambiguous sites and I kept the degenerate base code in analyses.

There were two well-supported clades in both the Bayesian and ML gene trees (Fig. 2.2, Fig. A1). One clade contained the domestic dog and gray wolf sequences plus one coyote sequence (AF541876) hypothesized to be from a coyote-dog hybrid (Adams et al. 2003b). The second clade contained all of the Great Lakes wolf and red wolf sequences, the remaining coyote sequences, and one gray wolf sequence (AY812740) believed to be a Mexican gray wolf-coyote hybrid (Leonard et al. 2005). The three novel aDNA sequences generated in this study grouped in the second clade. Nodal support within these two clades was generally low, as is expected for closely related taxa, but two of the aDNA samples were sister to each other with moderate support (0.92 posterior probability, 0.76 bootstrap support). These two clades, Old World gray wolf/dog and New World coyote/red wolf/eastern wolf/Great Lakes wolf, are

well supported in the literature (Roy et al. 1996, Vilà et al. 1999, Leonard et al. 2002, Adams et al. 2003a, Hailer and Leonard 2008, Fain et al. 2010, Rutledge et al. 2010).

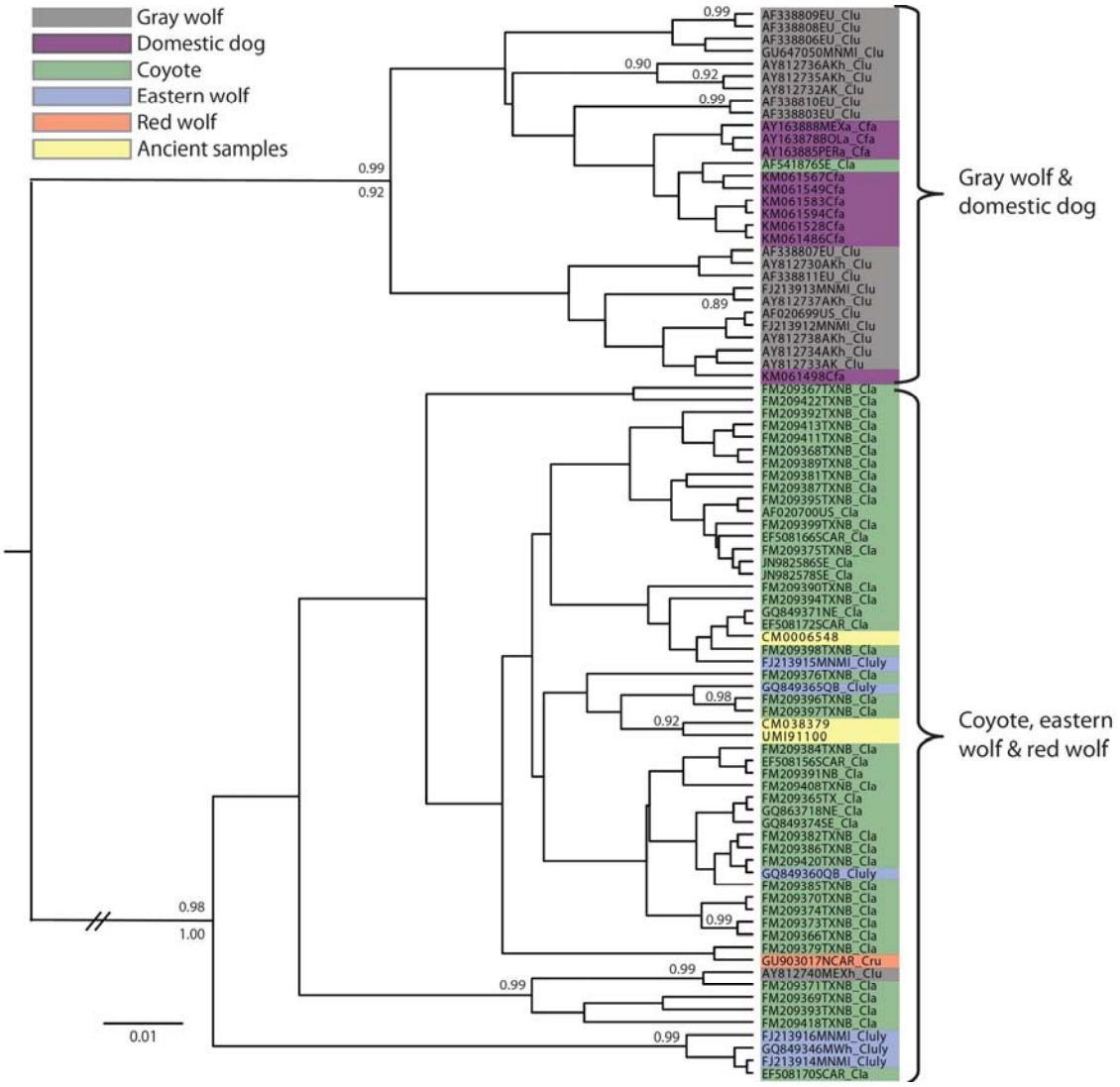

Figure 2.2. Gene tree showing the relationships among canid mitochondrial control region sequences. Bayesian posterior probabilities above 0.90 are listed above the branches and maximum likelihood bootstrap values above 0.90 are listed below the branches. Each color represents a different species. Tip names include the Genbank accession number assigned to each sequence followed by a geographic sampling location, if available, and an abbreviated species name. Historic and ancient DNA sequences downloaded from Genbank are indicated by 'h' and 'a,' respectively. The ancient DNA sequences generated in this study are named according to their museum accession numbers as in Table 1. Other abbreviations are as follows: Clu, *Canis lupus*; Cfa, *Canis familiaris*; Cru, *Canis rufus*; Cla, *Canis latrans*; Cluly, *Canis lupus lycaon*; EU, Europe; MEX, Mexico; BOL, Bolivia, PER, Peru; QB, Quebec; US, United States; AK, Alaska; MNMI, Minnesota and Michigan; MW, Midwest USA; NE, New England, USA; SE, Southeast USA; TXNB, Texas and Nebraska, NB, Nebraska; SCAR, South Carolina; NCAR, North Carolina.

DISCUSSION

I detected three novel aDNA haplotypes that clearly grouped with the New World canid clade, rejecting the hypothesis that gray wolves were dominant in the southeast 500-2,000 years ago. Within the New World mtDNA clade, the sequences I generated did not group closely with extant red wolf mtDNA haplotypes or the unique Algonquin eastern wolf cluster, suggesting they are not part of a monophyletic eastern canid lineage. The two aDNA samples sister to each other grouped closely with haplotypes found in multiple other eastern canids, including northeastern Great Lakes wolves, eastern wolves, and southeastern coyotes (Hailer and Leonard 2008, Leonard and Wayne 2008, Rutledge et al. 2010), although nodal support was low. Similarly, the third novel aDNA haplotype clustered within coyote haplotypes found throughout the southeastern United States. This lack of geographic structure is consistent with other canid mtDNA studies that document little phylogenetic structuring of coyotes or gray wolves, a probable outcome of their high mobility (Vilà et al. 1999, Koblmüller et al. 2012).

There are three plausible origins for the haplotypes I identified. First, my sequences could be from coyotes, which would indicate that coyotes were present in the southeastern United States continuously instead of intermittently as previously suggested. Although coyotes could have been present in the southeastern United States 350-1,900 years ago, the size of the three teeth samples I analyzed was more wolf- than coyote-like (Nowak 2002). If morphological analyses are correct, it is more likely the teeth samples I analyzed represent a wolf.

Given the size of the teeth I sampled, the haplotypes I recovered may alternatively be the result of historic hybridization between coyotes and a wolf species (gray or red), leading to introgression of coyote haplotypes into the southeastern United States wolf population (Roy 1996). A similar scenario was observed both in eastern wolves **(**Wilson et al. 2003, Wheeldon

and White 2009, Rutledge et al. 2010) and Great Lakes wolves (Koblmüller et al. 2009), which hybridized with coyotes when coyotes expanded their range during Pleistocene glacial and post glacial periods, resulting in coyote mtDNA haplotypes in modern wolf populations. If the ancient samples I analyzed here represent coyote-gray wolf hybridization events, some degree of hybridization occurred in the southeastern United States earlier than 287-430 years ago, as proposed by vonHoldt et al. (2011). Additionally, given the age of my samples (350-1,900 years old), historic hybridization would likely have been due to natural events or early human activities, not landscape changes associated with European colonization. Under these circumstances, coyote-wolf hybrids may have occupied the southeastern United States for a long time, filling an important niche as a large predator (Roy et al. 1996).

If my samples represent an ancient coyote-red wolf hybridization event, it would also suggest coyote-red wolf hybridization has been a continuous and likely dynamic process up to the present day. Interestingly, canid hybridization is often unidirectional (i.e., female coyotes mate with male wolves; Lehman et al. 1991), which may explain why coyote mtDNA is found in putative eastern and red wolf populations and generally not the other way around. Yet with contemporary red wolves, females and males both hybridize with coyotes, although it may still be biased toward female coyotes (Hailer and Leonard 2008, Bohling and Waits 2015, Hinton et al. 2015), complicating hybridization patterns between the two groups. Additional analyses focused on Y-chromosome or nuclear genes in ancient samples would provide information regarding the paternal lineage (Hailer and Leonard 2008, Wilson et al. 2012, Bohling and Waits 2015), but such comparisons were beyond the scope of this study given the limited quantity and degraded quality of the aDNA.

Lastly, my historic samples could represent red wolves, a lineage that may be closely related to coyotes (Wilson et al. 2000, Hedrick et al. 2002, Chambers et al. 2012). Incomplete

lineage sorting may explain why mtDNA haplotypes from ancient red wolves cluster with coyote mtDNA haplotypes, and not closely with extant red wolves. If coyotes and red wolves diverged from a common ancestor (Chambers et al. 2012), my aDNA sequences may represent shared ancestral haplotypes that have since been lost from contemporary red wolves, a possible result of population bottlenecks and inbreeding (Brzeski et al. 2013). Incomplete lineage sorting is common in recently diverged populations and species, and prevents reciprocal monophyly (Degnan and Rosenberg 2009). Others have observed this pattern within the Old World gray wolf/domestic dog clade (Vilà et al. 1999, Leonard et al. 2002), as I have observed in the present study. There are other examples of distinct mammal species displaying paraphyletic mtDNA phylogenies, such as brown bears (*Ursus arctos*), polar bears (*Ursus maritimus*; Cronin et al. 1991), mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*; Cronin et al. 1988). While my study is limited in geographic scope and sample size, it is possible that my data indicates relatively recent divergence between red wolf and coyote rather than hybridization. However, distinguishing incomplete lineage sorting from other hypotheses such as hybridization is difficult and requires more data than I have collected here.

Based on my results, red wolves may represent an evolutionary unit of conservation value, either as an ancient hybrid or as a unique lineage (Allendorf et al. 2001). These data suggest that a contemporary hybrid event was not the origin of red wolves. Hybridization is recognized as a natural evolutionary process and a facilitator of speciation (Mallet 2007); if red wolves have an ancient hybrid origin, it would not preclude the species from protection, and furthermore, it emphasizes the dynamic nature of canid evolution. If red wolves are a unique, independent lineage, they represent the only endemic wolf species in the United States, a species that is currently on the brink of extinction in the wild. Additional historic

samples from a larger geographic area will certainly help to clarify canid taxonomy in the southeastern United States, particularly if obtained sequences align closely with red wolves or the ancient haplotypes presented here. In the meantime, any plans to remove protection for red wolves would be premature.

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CHAPTER 3: INBREEDING AND INBREEDING DEPRESSION IN ENDANGERED RED WOLVES (*Canis rufus*)

INTRODUCTION

Inbreeding depression, the reduction in offspring fitness caused by mating among close relatives (Allendorf and Luikart 2007), is widespread in small, wild populations and is a major concern in conservation biology because it can directly affect population persistence (Crnokrak and Roff 1999, Keller and Waller 2002). The negative effects of inbreeding depression in wild populations are well documented in a diversity of taxa, from insects (Saccheri et al. 1998, Franke and Fisher 2013), fish (Ala-Honkola et al. 2009, Naish et al. 2013), and birds (Keller 1998, Townsend et al. 2009, Grueber et al. 2010), to small (Gage 2006, Nielsen et al. 2012) and large mammals (Coltman et al. 1999, Dunn et al. 2011, Walling et al. 2011). Harmful effects of inbreeding are attributed to an increase in genome-wide homozygosity resulting in the expression of deleterious recessive alleles (dominance hypothesis) and/or loss of heterozygous advantage (overdominance hypothesis; Charlesworth and Wallis 2009). There is evidence to support both processes, but expression of deleterious alleles appears to be the most common cause of inbreeding depression (Charlesworth and Charlesworth 1999, Keller and Waller 2002).

Recessive mutations will only cause inbreeding depression if they occur at gene(s) affecting fitness and result in a lower fitness than the general population (Allendorf and Luikart 2007). A population may, by chance, have few deleterious alleles at adaptive loci because of founder effects or genetic drift (Lacy et al. 1996, Keller and Waller 2002). When this happens, the expression and severity of inbreeding depression may vary or escape notice.

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Lacy et al. (1996) found that inbred lines of mice (*Peromyscus spp.*) exhibited reduced fitness in different traits and varying levels of severity as a consequence of random founder effects. Genetic purging, the removal of deleterious alleles through natural selection, can also influence the expression and severity of inbreeding depression (Lacy and Ballou 1998). However, in theory, no population is invulnerable to the deleterious effects of inbreeding, making it a major concern for endangered species management (Lacy 1997, Saccheri et al. 1998, Crnokrak and Roff 1999, O'Grady et al. 2006).

A complete understanding of the consequences of inbreeding in wild populations requires robust and direct measures of relatedness, and careful, long-term measures of reproductive success and survivorship (Pemberton 2004, Szulkin et al. 2007). Heterozygosity values calculated from multi-locus genotype data have been used to evaluate inbreeding depression in wild populations but are not ideal because they do not directly measure inbreeding (Pemberton 2008, Szulkin et al. 2010, Taylor et al. 2010, Grueber et al. 2011). Multigenerational pedigrees that map relatedness of breeding individuals are preferred, but such studies are generally rare as pedigrees are uncommon and long-term life history data on wild populations is often lacking. Therefore, species, such as the red wolf (*Canis rufus*), for which inbreeding and fitness data are available serve as model organisms because they reveal the influence of inbreeding and inbreeding depression in wild populations (Keller 1998).

Red wolves are critically endangered canids endemic to the southeastern United States (Phillips and Parker 1988, Nowak 2002, Hinton et al. 2013). Although once abundant throughout the southeast, persecution and habitat loss confined red wolves to Louisiana and Texas where they suffered from high levels of parasitism and hybridization with coyotes (*Canis latrans*; Paradiso and Nowak 1972, Custer and Pence 1981, Phillips et al. 2003). The threat of extinction

in situ led the United States Fish and Wildlife Service (USFWS) to bring the remaining individuals into captivity in the mid to late 1970s and establish a captive breeding program, after which red wolves were declared extinct from the wild in 1980. Fourteen individuals eventually became the founders of all present day red wolves, although only 12 are represented genetically in the current population (Riley and McBride 1975, Phillips and Parker 1988, Phillips et al. 2003, USFWS 2013). Starting in 1987, red wolves were reintroduced to Alligator River National Wildlife Refuge in northeastern North Carolina, where the population has grown since reintroduction (Philips et al. 2003, Hinton et al. 2013). The USFWS Red Wolf Recovery Program has maintained detailed records, including reproductive histories, birth dates, causes of death, pack composition, and a population-wide pedigree.

In the captive red wolf population, increased levels of inbreeding are correlated with decreased litter size, but lethal equivalents are near zero suggesting minimal inbreeding depression has occurred relative to other inbred canids (Kalinowski et al. 1999, Rabon and Waddell 2010). Current management procedures include deliberately pairing captive red wolves to reduce inbreeding and maximize genetic diversity (Waddell and Long 2013) thus, the results of inbreeding depression studies from captive wolves may not reflect the potentially high levels of inbreeding found in the wild population where wolves are free to choose mates. For instance, wild Scandinavian gray wolves (*Canis lupus spp.*) have large inbreeding coefficients that are correlated with decreased pup survival (Liberg et al. 2005). This result is consistent with other captive and wild wolf populations where clear associations exist between inbreeding and blindness, reduced reproductive success, decreased litter size, reduced sperm quality, and congenital bone deformities (Laikre and Ryman 1991, Laikre et al. 1993, Asa et al. 2007, Räikkönen et al. 2009).

Wolves may be able to avoid the deleterious effects of inbreeding depression by choosing unrelated individuals as mates, a behavior that has been documented in a number of wild wolf populations (Sillero-Zubiri et al. 1996, Smith et al. 1997). Reintroduced Yellowstone gray wolves nearly completely avoid inbreeding despite a small founding population (vonHoldt et al. 2007). There is evidence of inbreeding avoidance in wild red wolves as well (Sparkman et al. 2012a), but because the wild red wolf population is small and isolated, inbreeding may be unavoidable if background levels of relatedness are high. Given potential problems associated with inbreeding depression, an assessment of inbreeding and associated fitness costs in the wild red wolf population is warranted. More broadly, the red wolf pedigree and long-term data provide a rare opportunity to evaluate inbreeding and inbreeding depression in a long lived carnivore, and contributes to the broader understanding of the patterns and effects of inbreeding in wild populations. My objectives were to evaluate: 1) the degree to which inbreeding has increased since red wolf reintroductions, 2) the number of lethal equivalents (a standardized measure of inbreeding depression), and, 3) the effect of inbreeding on fitness-related traits.

METHODS

Study population. I used 23 years of data collected from the reintroduced wild red wolf population. Red wolf reintroduction efforts began in 1987 with the release of 4 adult wolf pairs at Alligator River National Wildlife Refuge (ARNWR) in northeastern North Carolina (Philips et al. 2003, Hinton et al. 2013). From October 1987–November 1994 an additional 60 wolves were intermittently released to bolster the new population; wolves were released either as pairs, sibling groups, or family groups (pers comm. Art Beyer, USFWS). By 1994, the wild population was self-sustaining via wild births, although occasional cross-fostering of captive born pups into wild litters continues to the present. Since the original reintroductions, the recovery area has grown to

encompass 1.7 million acres throughout 5 counties (Dare, Tyrrell, Hyde, Beaufort, and Washington), and the red wolf population has increased to about 100 individuals (USFWS 2013).

USFWS biologists closely monitor red wolf reproduction, mortality, home-range, and pair-affiliation with bi-weekly aerial flights and radio telemetry (Phillips et al. 2003, USFWS 2013). Each year's juveniles are target trapped and fitted with radio-collars; adults are recaptured when radio-collars need to be replacement. Wolves are captured with soft-catch, off-setting foothold traps, during which USFWS biologists take genetic samples and record morphological measurements and overall health. When a radio-collar mortality signal is detected, biologists attempt to collect the wolf and assess cause of death. USFWS biologists also search out denning red wolf pairs to determine litter size, implant transponders, and take genetic samples from pups each spring.

Due to coyote range expansion eastward into the recovery area, coyote-red wolf hybridization was first documented in 1993 (Phillips et al. 2003). Hybridization is considered a major threat to red wolf recovery and prompted development of an adaptive management strategy to prevent further introgression of coyote genetic material into the wild red wolf population (Kelly et al. 1999, Stoskopf et al. 2005, Rabon et al. 2013). Under the adaptive management plan, a genetic based maximum likelihood approach was designed to identify hybrids and assign red wolf ancestry (see Miller et al. 2003 for genetic classification details); animals considered to be greater than or equal to 87.5% red wolf were allowed to remain in the wild population (Stoskopf et al. 2005). I followed the USFWS criteria and treated all animals determined to be least 87.5% red wolf as part of the wild red wolf population. Part of the adaptive management plan also included sterilizing coyote and hybrid mates, so some red wolves

had sterile mates for parts of their reproductive years, which I accounted for in the analyses (see **Reproductive success**).

Pedigree. The red wolf pedigree was previously constructed from extensive field data and verified with genetic analyses (Adams 2006). Briefly, red wolves were genotyped at 18 microsatellite loci; multilocus genotypes were used to confirm parentage determined from field data and assign parentage to individuals with unknown pedigrees (Miller et al. 2003, Adams 2006). Parentage could be successfully assigned at the 95% confidence level 95% of the time when one parent was known (~14% of cases) and 88% of the time when neither parent was known (~27% of cases); in most cases (~59%) both parents were identified through field information and verified via genetic methods (see Adams 2006 for details). All known red wolves were included in pedigree construction and calculation of inbreeding coefficients; percent red wolf ancestry was determined after parentage assignment for management purposes and to characterize hybridization events in the population (Miller et al. 2003, Adams 2006). In the pedigree, 90% of all ancestry is known. The pedigree includes 764 wild born red wolves; of these, at least one parent is known for 738 wolves, both the dam and sire are known for 685 wolves, and all four grandparents are known for 635 wolves. The pedigree spans almost 7 generations and is maintained in the program SPARKS (ISIS 2011). Inbreeding coefficients were derived from PMx software (Lacy et al. 2011); the pedigree inbreeding coefficient (f) was the probability that 2 copies of an allele were identical by descent; an individual was inbred if $f > 0$.

To assess pedigree complexity and visualize the potential inbreeding loops within the population I plotted the lineage of the first wild born breeding red wolf (studbook id=10344) and her mate (studbook id=10392) through time with R-package kinship2 (Therneau et al. 2014). The pair was representative of the entire pedigree in that their offspring encompassed the spread of

inbreeding coefficients observed. Non-breeding offspring were excluded from the plot for simplicity. I determined if the average *f* of wild born litters increased over time using linear regression.

Lethal equivalents. I estimated the number of lethal equivalents (LE) per haploid genome (β) for red wolf survival to 18 months (S_i) following Kalinowski and Hedrick's (1998) maximum likelihood method. Lethal equivalents are a standardized measure of the effect size of inbreeding depression in a population (Morton et al. 1956) and defined as the number of deleterious alleles in a haploid genome whose cumulative effect is equivalent to 1 LE (Allendorf and Luikart 2007).

Inbreeding depression analyses. Fitness is defined as the average number of offspring an individual contributes to the next generation and is calculated as the product of reproductive success and survivorship (Falconer 1960, Allendorf and Luikart 2007). Thus, to determine if red wolf fitness was influenced by inbreeding, I investigated whether parental or individual inbreeding coefficients predicted: lifetime number of litters (LNL), the average number of litters a wolf had per reproductive year (ANL), litter size, probability of becoming a breeder, adult survival, juvenile survival, and adult body size. To avoid underestimating inbreeding depression I only included animals in analyses if they were wild born in the recovery area and all 4 grandparents were known. I originally included red wolf ancestry (0 = introgressed ancestry, 1 = 100% red wolf) as an explanatory variable in my analyses because individuals with coyote ancestry could have experienced heterosis and suffered less from inbreeding depression (Grant et al. 2003). Alternatively, introgression could have caused outbreeding depression and reduced individual fitness (reviewed in Edmands 2007). However, I removed ancestry from all final models except those evaluating body sizes because it was not an important predictor of fitness,

substantially decreased sample sizes, and removing it did not qualitatively change results. Unless otherwise reported, models encompassed fitness data collected from 1989-2012; specific data constraints for each fitness variable are discussed in detail below.

Reproductive success. I estimated LNL and ANL by the number of litters an individual produced rather than the total number of offspring, because until 1999 dens were not consistently sampled and pups were not counted; instead breeding pairs and the presence or absence of litters were noted. To determine the effect of inbreeding on LNL and ANL I ran generalized linear mixed effect models (GLMM) using the R-package lme4 (Bates and Maechler 2010) with a log-link function and Poisson distribution. Only individuals that lived to reproductive age (18 months), had known death dates, or were suspected dead from field signs were included in the LNL models (n=168); all wolves that lived to reproductive age were included in ANL (n=201). For the models with LNL and ANL as response variables, explanatory variables were: *f*, years reproductively available (LNL only), years holding a territory (ANL only), sex, dam *f*, sire *f*, dam age, sire age, and presence of helpers at birth (yes or no) as fixed factors; litter ID (identifier for the litter in which the focal animal was born) and year born were random factors. I included random factors to control for non-independence between litter mates and variation in year born. Parental *f* and age were used to test if there was a parental effect on offspring reproductive success. The variable "years reproductively available" was included in all LNL models to account for years red wolves were paired with sterile mates and thus were unable to reproduce irrespective of inbreeding; years reproductively available was calculated based on the number of years a wolf was reproductively available minus the number of years paired with a sterile mate. Presence of helpers in a pack has been shown to affect red wolf reproductive success and was therefore included in models (Sparkman et al. 2011, Sparkman et al. 2012b). I defined the

presence of helpers at birth as the incidence of non-breeding pack members that participated in pup rearing.

I ran GLMMs with a logit-link function and binomial error distribution to determine if inbreeding affected the probability that a wolf became a breeder. I defined breeder status as 1 if a red wolf had at least one litter in its lifetime or 0 if it never bred. With breeder status as the response variable, the fixed and random explanatory variables were the same as LNL and ANL and only included individuals that lived to reproductive age (18 months) and had known or suspected death dates (n=168). I reran LNL, ANL, and probability of breeding models to evaluate if inbreeding depression differed when using a dataset that only included individuals born 2001 onward, the timeframe where the most inbred litters were born and litters were monitored more closely than previous years for management purposes.

I evaluated models with litter size as the response variable because inbreeding in the captive population was correlated with reduced litter size (Rabon and Waddell 2010). I only included litters where all pups were given transponders before becoming mobile, usually within approximately 2 weeks of parturition (n=105; pers comm. Art Beyer, USFWS). Although this removed litters from the early years of the program before dens were sampled and pups were fitted with transponders, it assured the most accurate litter counts available. I used GLMMs with a log-link function and Poisson distribution and the following explanatory variables: *f* of the litter, dam *f*, sire *f*, dam age, sire age, and year born as fixed factors and pair ID as a random factor. Each breeding pair was given a unique identity that was used instead of litter ID because pair ID accounted for different litters with the same parents. I excluded 4 litters that each had multiple sires and therefore lacked a single *f* value.

Survival. To examine the effect of inbreeding on survival, I ran Cox proportional hazard mixed effect models with the R-package coxme (Therneau 2013) with adult and juvenile survival as response variables; I defined juvenile survival as living to 18 months. Cox proportional hazard models estimate a baseline hazard function where the null expectation is equal to 1, meaning that parameter estimates greater than 1 increase the hazard of dying while estimates less than 1 increase the probability of survival. Cox models are useful because you can include individuals that outlive or are removed from the population during the specified survival timeframe [censored]. I censored individuals that were alive at the end of each survival period while individuals that died or were suspected dead with high confidence were uncensored; for both survival periods the terminal event was death, where age at time of death was calculated in days. I also included individuals that died due to anthropogenic or management causes but censored them, such that 0 = a censored individual (survived, removed from the population, or died due to anthropogenic causes), and 1 = an individual that died during the specified time window (adult survival: 0=178, 1=104, juvenile survival: 0=237, 1=36). Known causes of death included anthropogenic incidents (gunshots, vehicular accidents), management actions (trapping, injury, removal) and natural events (disease, interspecific conflict). Explanatory variables for both model sets included: *f*, sex, dam *f*, sire *f*, dam age, sire age, and territory (yes or no if a holder; adult survival only), as fixed factors and litter ID and year born as random factors. There were seven outlier sire *f* values (sire $f > 0.3$), thus I ran survival models with and without the outliers to evaluate model sensitivity. I also reran adult and juvenile survival models, like reproductive models, with a dataset truncated to only include individuals born 2001 onward.

Body size. I tested if inbreeding influenced body size because physical size can affect behaviors important to fitness, such as an individual's ability to secure a mate, effectively hunt,

or hold a territory. To create a single measure of overall body size I implemented a Principle Components Analysis (PCA) with measurements of body length, hind foot length, shoulder height, ear size, and tail length. PC1 encompassed 62% of the total variance, after which there was a precipitous drop in the variance explained by PC2-PC5. All morphometric variables were positively associated with each other, and based on individual loadings, each variable was important in PC1 (Table B1). Thus, PC1 effectively represented overall body size and was used as the response variable in models to evaluate the effect of inbreeding on red wolf body size (Fig. B2). I used linear mixed effect models with a Gaussian error distribution to evaluate the effect of inbreeding on body size. Explanatory variables were: *f*, sex, ancestry (0 = introgressed ancestry (any coyote ancestry), 1 = 100% red wolf), dam *f*, sire *f*, dam age, and sire age as fixed factors, and pair ID and year born as random factors; sex was included in every model to account for sexual dimorphism. Only measurements taken from fully grown wolves (> 10 months old) were used (n = 128); if individuals were captured multiple times as adults, I averaged their measurements.

I evaluated GLMM reproductive success and Cox proportional hazard adult survival models with PC1 as an explanatory variable to evaluate the relationship between body size and fitness, similarly to methods in Sparkman et al. (2011). I also evaluated if PC1 predicted the probability of holding a territory, an important component of annual reproductive success (Table 3.1). GLMMs with a logit-link function and binomial error distribution were run with territory (0 = never held a territory, 1 = held a territory for at least one breeding season) as the response variable, fixed explanatory variables included PC1, sex, dam age, sire age, and an interaction between PC1 and sex, and litter ID as a random variable.

Model selection. Initial data exploration for all analyses followed Zuur et al. (2010), where collinearity, independence, heterogeneity, interactions, normality, and the influence of outliers was examined for each model set. Individual *f* was correlated with parental inbreeding coefficients and parental ages were correlated with each other, thus these variables were not included in the same models (Table B2). I also confirmed that year of birth was not a confounding variable or directly correlated with fitness variables. All models were ranked with AIC_c and AIC_c weight (w_i; Burnham and Anderson 2002) and validated by examining residuals and fitted values as suggested by Zuur et al. (2009). I averaged models encompassing 95% of AIC_c w_i using the natural-average method (Burnham and Anderson 2002) in R package MuMIn (Bartoń 2009). I standardized input variables in R package arm (Gelman et al. 2009) to rank explanatory variables and directly compare the effect size of model-averaged coefficients (Grueber et al. 2011). Model averaging was used because it takes model selection uncertainty into account and provides methods to evaluate the relative importance of each variable. Relative importance was calculated by summing AIC_c w_i across all models where a variable occurs in the final model set. Larger values indicate that a variable is more important relative to other variables in explaining variance in the response variable (Burnham and Anderson 2002). I report model averaged coefficients, unconditional standard errors which incorporate model selection uncertainty, and relative variable importance.

RESULTS

All but the first wild born red wolf (studbook id=10344) had inbreeding coefficients greater than zero (mean $f = 0.154$, range 0 - 0.383; Fig. 3.1). Out of all wild breeding pairs, fourteen had litters with $f \geq 0.25$, producing 102 highly inbred wolves with inbreeding coefficients above the equivalent of sibling-sibling/parent-offspring matings (n=31 litters). The

most inbred individuals ($f > 0.28$) were from litters born 2001-2012, more than half of which were from 2008-2012. A large percent of the population (85.1%) was either low to moderately inbred at $0 < f < 0.125$ (N=290 from 86 litters) or had high levels of inbreeding at $0.125 \leq f < 0.25$ (N=293 from 67 litters). There was no difference between average male ($f = 0.154$) and female ($f = 0.156$) inbreeding coefficients ($t = 1.65$, df = 673, $P = 0.47$). Since reintroductions, the average inbreeding coefficients of litters increased significantly from 0.031 in 1988 to 0.169 in 2012 ($F = 82.78$, df = 23, $P < 0.001$; Fig. 3.2). I estimated β (the number of LE per haploid genome) on juvenile survival to 18 months as 0.00.

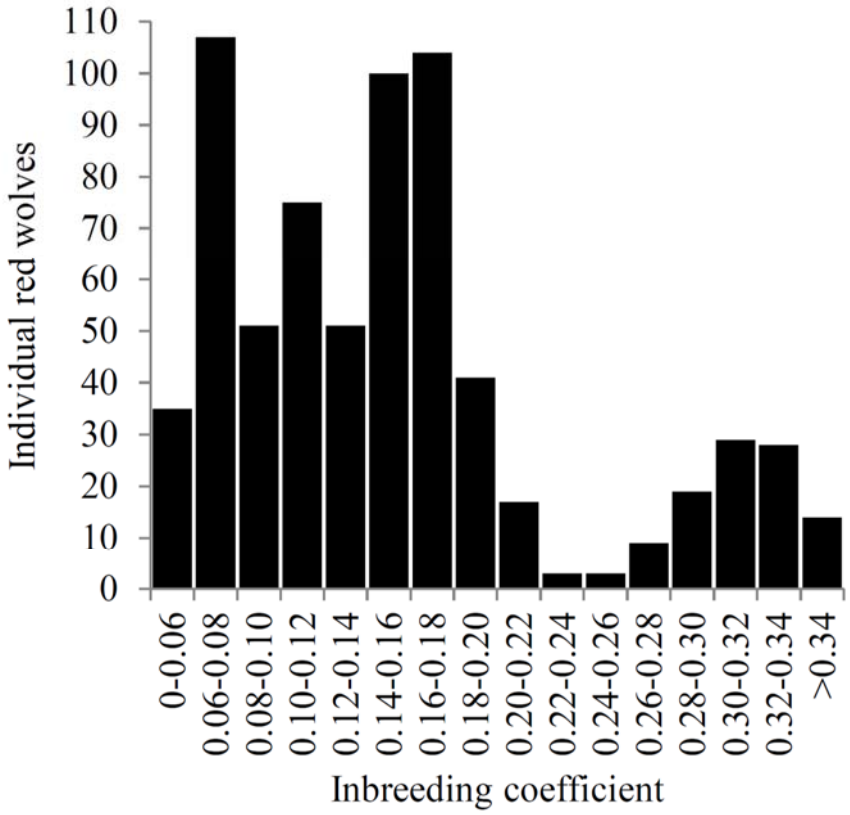

Figure 3.1. Distribution of inbreeding coefficients for wild born red wolves (*Canis rufus*) of known parentage (N=685).

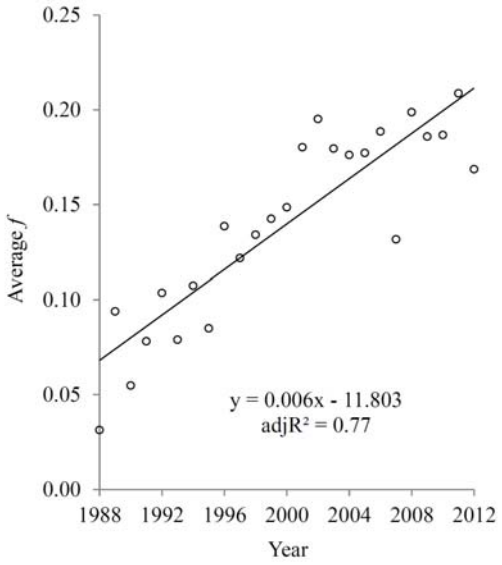

Figure 3.2. Average yearly inbreeding coefficients (*f*) for wild born red wolf (*Canis rufus*) litters (n=182) since 1988.

The ancestry plot of red wolf 10344 and her mate, 10392, revealed a complex pedigree with numerous inbreeding loops (Fig. 3.3). Although 10344 was the only wild born wolf with *f* = 0.0 (her parents were unrelated), the kinship between the pair was 0.102; this was likely due to background levels of relatedness in captive breeding prior to reintroductions (the pair shared the same maternal grandmother as well as 10392's parents were half aunt/half nephew). There were also a number of matings among close relatives resulting in the highest inbreeding coefficients observed (Table 3.1).

Table 3.1. Known relationships for red wolf (*Canis rufus*) breeding pairs resulting in offspring with inbreeding coefficients (*f*) >0.19.

Relationship category	Breeding pairs
1st cousin	7*
Half uncle/niece	2
Uncle/niece	4
Aunt/nephew	2
Half sibling	1
Full sibling	5

*In three of the 1st cousin breeding pairs, one mate had full sibling parents and one cousin pair was 1st cousins from both parents.

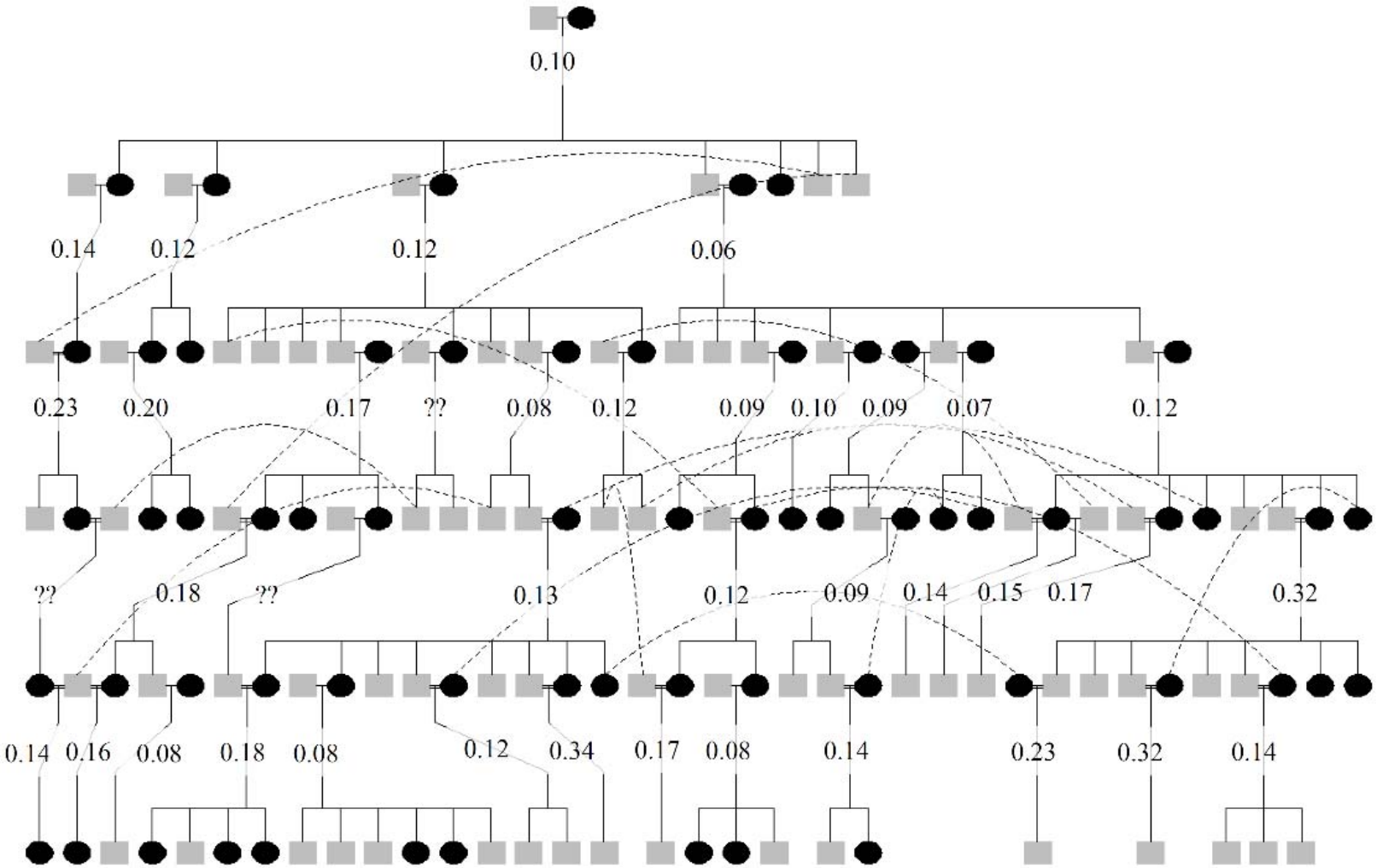

Figure 3.3 Pedigree plot of one of the first wild born red wolf (*Canis rufus*) breeding pairs; circles denote females, squares denote males, and the dashed line connects the same individual present multiple times throughout the pedigree, first as offspring and later as a breeder. The kinship of each pair (and resulting inbreeding coefficient of their offspring) is displayed below the pair; ?? is indicative of unknown parents or grandparents. Non breeding offspring were excluded for simplicity.

Inbreeding depression varied by trait; body size was strongly affected by inbreeding, whereas reproductive and survival traits were only minimally affected by inbreeding. In body size models, individual *f* was negatively associated with overall size. This relationship was strongly supported given that 95% confidence intervals did not overlap zero and *f* was highly ranked in the average model set (Table 3.2). Red wolf ancestry, parental ages, and parental *f* values were not influential in body size models (Table 3.2). I found body size did not affect the fitness measures I evaluated, similarly to Sparkman et al. (2011), but body size was positively associated with the probability of holding a territory (Table B1, Fig. B3).

Inbreeding depression was less evident in reproductive success, although in ANL GLMM models individual *f* and dam *f* had high relative importance and negatively affected ANL (Table 3.2). Sire *f* was also negatively associated with ANL but had low relative importance. GLMM models evaluating the probability of becoming a breeder were similar to ANL in that individual *f* and dam *f* adversely affected the probability of breeding, although both were relatively less important than they were in ANL models (Table 3.2). Sire *f* was positively correlated with the probability of breeding but was the lowest ranked variable in the models. Sire *f*, dam *f*, and individual *f* all negatively influenced LNL but had the lowest relative importance in LNL models (Table 3.2).

The final GLMM model set for litter size encompassed the null model and thus provided little support for inbreeding depression. Model inferences for all models of reproductive success, with data truncated to wolves born 2001 onward, were comparable to the full data models.

Inbreeding depression was less evident in reproductive success, although in ANL GLMM models individual *f* and dam *f* had high relative importance and negatively affected ANL (Table 3.2). Sire *f* was also negatively associated with ANL but had low relative importance.

Table 3.2. Parameter estimates (β), unconditional standard error (SE), 95% confidence limits (CL), and relative importance (RI) of variables in the final averaged models evaluating lifetime number of litters (LNL), annual number of litters (ANL), the probability of becoming a breeder, litter size, and body size in endangered wild red wolf (*Canis rufus*).

Dependent variable	A priori models	Explanatory variable	β	SE	CL	RI
LNL	28	Years reproductively available	2.17	0.19	1.79, 2.54	1.00
		Helper	-0.40	0.21	-0.81, 0.02	1.00
		Dam age	0.40	0.15	0.10, 0.70	1.00
		Sire age	-0.36	0.18	-0.70, -0.02	0.52
		Sex (m)	-0.22	0.15	-0.52, 0.07	0.32
		Sire f	-0.16	0.18	-0.50, 0.19	0.24
		Dam f	-0.12	0.15	-0.41, 0.16	0.23
		f	-0.05	0.20	-0.44, 0.34	0.20
ANL	30	Years with a territory	0.37	0.07	0.24, 0.50	1.00
		Helper	-0.15	0.09	-0.33, 0.03	0.49
		f	-0.07	0.07	-0.21, 0.07	0.41
		Dam f	-0.07	0.07	-0.21, 0.07	0.40
		Dam age	0.09	0.07	-0.04, 0.22	0.37
		Sire f	-0.06	0.07	-0.19, 0.07	0.19
		Sire age	0.03	0.07	-0.11, 0.17	0.16
		Sex (m)	-0.03	0.06	-0.16, 0.09	0.09
Probability of breeding	28	Years reproductively available	3.05	0.48	2.12, 3.99	1.00
		Helper	-0.75	0.55	-1.82, 0.33	1.00
		Dam age	0.36	0.40	-0.43, 1.16	0.28
		Sex (m)	-0.42	0.40	-1.20, 0.35	0.27
		f	-0.11	0.41	-0.91, 0.68	0.23
		Dam f	-0.13	0.45	-1.00, 0.74	0.21
		Sire age	-0.06	0.42	-0.88, 0.75	0.19
		Sire f	0.07	0.39	-0.70, 0.84	0.15
Litters*	27	Dam age	-0.20	0.11	-0.42, 0.02	0.60
		litter f	-0.12	0.13	-0.38, 0.13	0.27
		Dam f	0.01	0.12	-0.22, 0.24	0.19
		Sire age	-0.01	0.11	-0.24, 0.21	0.18
		Sire f	-0.05	0.13	-0.30, 0.20	0.15
		Year born	-0.06	0.12	-0.29, 0.16	0.09
Body size	18	Sex (m)	2.26	0.21	1.86, 2.67	1.00
		f	-0.98	0.36	-1.69, -0.28	0.87
		Ancestry	-0.40	0.34	-1.06, 0.26	0.24
		Dam age	-0.33	0.27	-0.86, 0.20	0.19
		Sire age	-0.09	0.28	-0.64, 0.46	0.09
		Sire f	-0.29	0.29	-0.86, 0.28	0.02
		Dam f	-0.14	0.37	-0.86, 0.57	0.02

* Denotes response variable where final model set encompassing 95% of AIC weight included the null model.

GLMM models evaluating the probability of becoming a breeder were similar to ANL in that individual *f* and dam *f* adversely affected the probability of breeding, although both were relatively less important than they were in ANL models (Table 3.2). Sire *f* was positively correlated with the probability of breeding but was the lowest ranked variable in the models. Sire *f*, dam *f*, and individual *f* all negatively influenced LNL but had the lowest relative importance in LNL models (Table 3.2). The final GLMM model set for litter size encompassed the null model and thus provided little support for inbreeding depression. Model inferences for all models of reproductive success, with data truncated to wolves born 2001 onward, were comparable to the full data models.

No inbreeding depression was observed in adult or juvenile survival (Table 3.3); the final Cox proportional hazard model set evaluating juvenile survival encompassed the null model, and individual *f* and dam *f* had little relative importance in either survival period. However, for adult Cox proportional hazard models, sire *f* was negatively associated with hazard (Table 3.3), such that an individual with an average inbred sire (sire $f = 0.154$) was 2.932 [exp(6.984*0.154)] times more likely to survive compared to an individual with an outbred sire. This was a strong relationship, where the 95% confidence interval of sire *f* did not overlap zero. When I tested model sensitivity by removing the seven most inbred sires (sire $f > 0.30$), sire *f* was no longer an important factor in survival, suggesting the relationship was driven solely by the outlier sires (Table 3.3). Similarly to reproductive success, adult survival analyses with full data were qualitatively similar to models run with data truncated to wolves born 2001 onward; when sires with $f > 0.3$ were removed, dam *f* increased in relative importance from 0.08 to 0.22 but 95% confidence intervals still overlapped zero. All final juvenile model sets based on truncated data encompassed the null model.

Other variables important to predicting reproductive success and survival included parental age, years of reproductive availability, years with a territory, presence of helpers, and sex. Longer lived red wolves, red wolves with a territory, and individuals with older dams had higher LNL, ANL, a greater probability of breeding, and increased survival. In contrast, sire age, and presence of helpers negatively affected reproductive traits and survival except for ANL and adult survival models (Tables B4-B10).

Table 3.3. Cox proportional hazard results from models evaluating adult and juvenile survival (survival to 18 months) in endangered wild red wolf (*Canis rufus*). Effect size, unconditional standard error (SE), 95% confidence limits (CL), and relative importance (RI) of variables are reported from the final averaged models; (-) indicates a variable was not in the final average model set. Effect size refers to the influence a parameter has on the proportional survival hazard where positive parameter estimates increase the hazard of dying while negative estimates increase survival.

		All data				Sire *f* values >0.30 removed			
	Explanatory	Effect	SE	95% CL	RI	Effect	SE	95% CL	RI
Adult survival	Territory	-2.70	0.37	-3.4, -2.0	1.0	-2.72	0.37	-3.5, -2.0	1.0
	Sire *f*	-6.98	3.05	-13.0, -1.0	0.8	-1.55	4.71	-10.8, 7.7	0.1
	Sire age	-0.12	0.10	-0.3, 0.1	0.4	-0.12	0.09	-0.3, 0.1	0.3
	f	-2.87	2.20	-7.2, 1.4	0.1	-1.62	2.25	-6.0, 2.8	0.2
	Sex (m)	0.22	0.25	-0.3, 0.7	0.0	0.25	0.25	-0.2, 0.8	0.1
	Dam age	-	-	-	-	0.06	0.09	-0.1, 0.2	0.1
	Dam *f*	-	-	-	-	1.70	3.51	-5.1, 8.6	0.1
Juvenile survival*	Sire *f*	-6.49	6.56	-19.3, 6.4	0.4	-0.21	7.44	-14.8, 14.4	0.1
	Sire age	0.00	0.20	-0.34, 0.4	0.4	-0.02	0.18	-0.4, 0.3	0.3
	Sex (m)	0.01	0.39	-0.8, 0.8	0.3	0.00	0.38	-0.78, 0.7	0.2
	f	-0.35	4.09	-8.4, 7.7	0.1	0.83	4.24	-7.5, 9.2	0.3
	Dam *f*	3.20	5.06	-6.7, 13.1	0.1	3.82	4.96	-5.9, 13.5	0.1
	Dam age	0.00	0.14	-0.3, 0.3	0.1	0.04	0.14	-0.2, 0.3	0.1

* Denotes response variable where final model set encompassing 95% of AIC weight included the null model.

DISCUSSION

The deleterious effects of inbreeding are a serious consideration for small wildlife populations of conservation concern (Keller and Waller 2002, O'Grady et al. 2006, Wright et al. 2008). In red wolves, inbreeding has increased substantially since reintroductions in 1987, resulting in a population that is almost completely inbred. The observed level of inbreeding appeared to be the result of both high background levels of relatedness and a number of matings among close relatives (Fig 3.3, Table 3.1). Compared to other wild populations, red wolf inbreeding coefficients are high (Table 3.4). The mean red wolf *f* value of 0.154 is greater than half-sibling matings, and although other wild populations have individuals with high *f* values, few have a documented population mean as high as wild red wolves (Table 3.4). The wild mean *f* value was also much higher than the captive red wolf population mean of 0.076 (Waddell and Long 2013). Similarly, the percentage of inbred wild red wolves (99%) is greater than other reported wild populations (Table 3.4). Although cooperative breeding species, like wolves, often have mechanisms to avoid inbreeding (Pusey and Wolf 1996, Fadao et al. 2000, Jamieson et al. 2009, Sparkman et al. 2012a), inbreeding values have significantly increased through time, a result attributable to a small number of founders (n = 12) and a single population with no possibility of gene flow from other wild red wolves.

In contrast, the number of lethal equivalents ($\beta = 0.00$) detected for juvenile survival in red wolves was much lower than other captive and wild populations (Ralls et al. 1988, Kruuk et al. 2002, Liberg et al. 2005, O'Grady et al. 2006, Dunn et al. 2011). For example, the average number of haploid LE for juvenile survival is 2.3 in 38 captive species (Ralls et al. 1988) and 1.2 in 6 wild species (O'Grady et al. 2006); haploid LE as high as 12.1 have been documented in wild pronghorns (*Antilocapra americana*; Dunn et al. 2011).

Table 3.4. Species, inbreeding coefficient (*f*), mean *f*, percent of population inbred (% inbred), the fitness consequences of inbreeding, and the population status (wild/captive) from studies with pedigrees that evaluated the effects of inbreeding (see Keller and Waller 2002 for older research). Table focused on wild populations but included captive Mexican gray wolves for comparison.

Species	*f*	Mean *f*	% Inbred	Fitness consequences	Captive/Wild	Author
Mexican wolf	0-0.61	-	-	Reduced litter size	Captive	Hedrick and Fredrickson 2008
Scandinavian wolf	0-0.41	-	92.0%	Decreased pup survival, litter size	Wild	Liberg et al. 2005
Red wolf	0-0.38	0.154	99.0%	Reduced body size	Wild	Present study
Bighorn sheep	0-0.31	0.042	25.0%	Decreased survival of female lambs	Wild	Rioux-Paquette et al. 2010
African wild dogs	0-0.28	0.074	37.5%	Shorter lifespans	Wild	Spiering et al. 2011
Stewart Island robin	0-0.25	0.070	-	Little inbreeding depression found	Wild	Laws et al. 2010
Pronghorn	0-0.25	0.026	22.0%	Decreased fawn survival to weaning, birth mass, foot length, condition	Wild	Dunn et al. 2011
Red deer	0-0.25	0.007/0.01	22%/42%	Decreased birth weight, first year survival	Wild	Walling et al. 2011
Collared flycatchers	0-0.25	0.002	1.0%	Reduced hatching, fledging, juvenile survival, recruitment, and skeletal size	Wild	Kruuk et al. 2002
Great tit	0-0.25	0.004	3.0%	Reduced hatching, fledging, recruitment, production of grand offspring	Wild	Szulkin et al. 2007
Meerkats	0-0.13	0.078	44.0%	Decreased pup mass, hind-foot length, growth, juvenile survival	Wild	Nielsen et al. 2012
Yellowstone gray wolf	<0-0.08	0.000	3 related matings	None observed	Wild	vonHoldt et al. 2007

Yet, there are also examples of inbred populations with few LE, such as Red Cockaded Woodpeckers (*Piciodes borealis*) which suffer from inbreeding depression in both reproductive and survival traits but have haploid LE = 0.37 for first year survival (Daniels and Walters 2000, O'Grady et al. 2006). My results are consistent with Kalinowski et al. (1999) who found few LE for captive red wolf survival to 180 days and estimated the number of LE in 13 founders to be near zero.

Fitness consequences associated with inbreeding varied by trait where inbreeding depression was strongest for body size such that more inbred individuals were smaller. Conversely, no inbreeding depression was detected in reproductive and survival measures, a finding consistent with my observed values of zero for lethal equivalents. The lack of inbreeding depression in reproductive and survival traits was surprising because inbreeding depression is generally strongest for direct fitness traits, which are under greater selective pressure and exhibit more directional dominance (where dominant alleles affect a trait in the same direction, resulting in a difference in means between heterozygous and homozygous phenotypes (Barton and Keightley 2002)) than morphometric measures (Crnokrak and Roff 1995, Roff 1998, De Rose and Roff 1999). Red wolf body size did not influence fitness directly but it did increase the probability of having a territory, which is important for securing reproductive opportunities, suggesting that smaller body size influences fitness indirectly by reducing the probability of becoming a territory holder. A reduction in body size associated with inbreeding has also been detected in other wolf species, including Mexican (*C. lupus baileyi*) and Nordic gray wolves (Laikre 1999, Fredrickson and Hedrick 2002). Additional studies have documented similar correlations between inbreeding depression and body size or skeletal measures in non-canid species, which may have indirect effects on sexual selection, intraspecific competition, survival,

or fecundity (Fredrickson and Hedrick 2002, Kruuk et al. 2002, Wisely et al. 2008, Bolund et al. 2010, Dunn et al. 2011, Nielsen et al. 2012, Naish et al. 2013). Inbreeding may affect morphology more than previously thought (Wright et al. 2007), and may represent a cost effective way of measuring the effects of inbreeding *in situ*; although see Ibáñez et al. (2011) who found no inbreeding depression in morphology, suggesting the large variation observed in inbreeding effects may make it difficult to generalize a trait's response.

Other traits that influenced red wolf fitness included parental age and years reproductively available. The influence of parental age was most evident in LNL models, where individuals with older dams and younger sires had higher LNL. Generally, reproductive success decreases with maternal age (Rabon 2014), but older females have more parental experience (Mech 2000) and in some mammals have heavier offspring to compensate for smaller litters (Ericsson et al. 2001), both of which could increase offspring fitness (Curio 1983). The only fitness measures that were negatively associated with dam age in red wolves were litter size and adult body size, but confidence limits overlapped zero for both traits thereby limiting my ability to make inference. Sire age varied more by trait and was not as relatively important as dam age, possibly reflecting different reproductive strategies between sexes (Weimerskirch et al. 2000, Miller et al. 2003).

The number of years a red wolf was reproductively available also increased LNL and the probability of breeding. While this is an intuitive relationship (the more years an individual is able to breed the higher their reproductive success) it also demonstrates the negative impact that sterile coyote placeholders may have on red wolf reproductive success. Habitat conversion and fragmentation, combined with animal translocations have increased rates of hybridization across animal taxa (Rhymer and Simberloff 1996, Allendorf et al. 2001), and as demonstrated with red

wolves, managers face a challenge of maintaining reproductive output while preventing introgression (Miller et al. 2003, Allendorf and Luikart 2007). The use of sterile placeholder mates has been a successful management technique to reduce coyote hybridization and introgression with red wolves (Stoskopf et al. 2005, Rabon et al. 2013), but it also reduces the years an individual is reproductively available. Interestingly, red wolf ancestry had little influence on body size or fitness, suggesting coyote introgression did not strongly influence fitness. However, future work focused on coyote introgression is needed to fully understand the influence of hybridization on wild red wolves.

There are several potential reasons for the lack of lethal equivalents and inbreeding depression observed in traits other than body size. Genetic purging could have reduced the genetic load in red wolves such that deleterious alleles directly associated with fitness were purged, whereas alleles indirectly associated with fitness such as body size, persisted in the population (Lacy and Ballou 1998, Crnokrak and Roff 1995). Although genetic purging may be effective at removing deleterious alleles in some inbred populations (Ballou 1997, Reed et al. 2003), in general, genetic drift is a stronger force than purging selection in small populations such as red wolves (Hedrick and Kalinowski 2000). A founder effect may be a more likely explanation for the pattern of inbreeding depression I detected. Random sampling of alleles in founder lineages affects the severity of inbreeding depression in inbred mice (Lacy et al. 1996) and white pigs (Rodrigáñeza et al. 1998) where inbreeding depression was attributed to a few deleterious alleles, which were not carried by all founders. This may be true for red wolves given that 13 founders had few LEs and no LEs were detected for captive juvenile viability (Kalinowski et al. 1999). If all red wolf founders lacked deleterious recessive alleles at genes affecting fecundity as well as survival, then I may continue to see minimal inbreeding depression

at these fitness traits. However, standard errors and confidence limits for effect sizes of individual and parental *f* values were large, especially in survival models (Table 3). This could indicate there is a lack of statistical power to detect inbreeding depression. For instance, in Cox juvenile survival models, the upper range of the 95% confidence limits for dam *f* (-6.71, 13.11) and individual *f* (-8.36, 7.65) encompassed some of the more dramatic inbreeding depression values reported in the literature.

The absence of significant inbreeding depression in reproductive success and survival may also have been caused by the lack of outbred individuals for comparison. In captive Mexican wolves, minimal inbreeding depression was detected until individuals from 3 unrelated lineages bred and the resulting offspring had higher fitness than the inbred parental lineages (Fredrickson et al. 2007, Hedrick and Fredrickson 2008); inbreeding depression could not be detected without outbred individuals because there was too little variation in *f*. The lack of non-inbred red wolves in my study may have masked the most detrimental effects of inbreeding depression. Alternatively, the most detrimental effects of inbreeding depression may not yet be detectable because all of the highest *f* values (> 0.28) are from red wolf litters born 2001-2012, more than half of which were born after 2008. Although model inferences for all fitness variables were similar between the full and restricted dataset, it is possible that fitness consequences from highly inbred wolves born recently may be detected once complete life history data are collected.

Hedrick and Kalinowski (2000) suggest that the true effects of inbreeding are generally greater than those observed, not less. This may be true for wild red wolves, and given that inbreeding will likely continue to increase, inbreeding depression is a continued concern for red wolf viability. Population management aimed at reducing inbreeding and inbreeding depression is needed. Common genetic management techniques, such as genetic rescue, have been

successful with Florida panthers (Johnson et al. 2010), Mexican wolves (Fredrickson et al. 2007), and bighorn sheep (Hogg et al. 2006), and could be applied by introducing more distantly related individuals through cross-fostering wolves from the managed captive breeding program into wild litters. There have been 21 cross-fostering events since 2002; in each instance, the captive born cross-fostered pups were less inbred (mean $f = 0.074$) and had lower mean kinship values (MK = 0.095) than the wild born averages (mean $f = 0.154$, MK = 0.160). Future management practices could increase cross-fostering or release captive born juveniles with the aim of reducing overall inbreeding and mean kinship in the wild population.

Multigenerational pedigrees are rare in wild populations, (see Table 4), but can provide unique insights into processes that influence inbreeding. For example, in a population of highly social African wild dogs (*Lycaon pictus*), high levels of inbreeding were attributable to a single pack (Spiering et al. 2011). This contrasts with results from meerkats (*Suricata suricatta*) where inbreeding was not the result of a few inbred family groups but was influenced by social dominance and was ubiquitous throughout the population (Nielsen et al. 2012). Red wolves live in social family groups, and similarly to meerkats, inbreeding was spread throughout the population. However, meerkats may tolerate low levels of inbreeding because the benefits of securing a breeding opportunity, even if with a related mate, outweigh the cost of inbreeding depression (Nielsen et al. 2012), unlike red wolves where inbreeding was likely the result of few founders and a closed population; an inherent problem facing any extremely small or endangered population.

The pervasiveness of inbreeding in wild populations is well recognized (Keller and Waller 2002), but factors influencing the extent of inbreeding depression are still being evaluated. My results demonstrate that inbreeding depression varies substantially by trait,

highlighting the need to evaluate a number of different fitness parameters/traits when examining inbreeding depression. While inbreeding significantly reduced red wolf body size, its influence on direct measures of red wolf fitness appears to be weak. With continued monitoring and pedigree construction in wild red wolf populations, the efficacy of genetic purging and prevalence of founder effects as individuals continue to become more inbred can be evaluated.

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CHAPTER 4: INFECTIOUS DISEASE AND RED WOLF CONSERVATION: ASSESSMENT OF DISEASE OCCURRENCE AND ASSOCIATED RISKS

INTRODUCTION

Wildlife disease epizootics, or epidemics, are becoming an urgent issue for the conservation and management of threatened and endangered species (Daszak 2000; Smith et al. 2009). For instance, disease outbreaks have contributed to several near extinctions and population crashes (see references in Woodroffe 1999; de Castro and Bolker 2004), directly and indirectly threatening wildlife populations by killing hosts faster than replacement, an outcome that makes small populations vulnerable to stochastic extinction (Woodroffe 1999). Generalist pathogens may pose the greatest risk to threatened wild populations because they can remain at high prevalence in numerous host species, lowering a pathogen's density threshold for transmission in small populations, which themselves are not dense enough for disease transmission (Lyles and Dobson 1993; Woodroffe 1999). The threat of infectious disease and pathogen-mediated population declines is compounded in threatened and endangered populations because they are small and often lack the genetic variability necessary to combat virulent pathogens (Spielman et al. 2004), making disease monitoring a necessary component of conservation programs.

Threatened and endangered populations can be especially vulnerable to disease that is transmitted by common, wide ranging species (Murray et al. 1999). For example, the catastrophic canine distemper virus (CDV) epizootic in wild endangered black-footed ferrets (*Mustela nigripes*) was likely transmitted by sympatric coyotes or badgers (*Taxidea taxus*—Williams et al. 1988).

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Generalist viral pathogens like CDV or rabies are most often responsible for disease-driven population declines, but other pathogenic groups, such as bacteria, helminths, arthropods, or protozoa, can also be detrimental for small populations (Pedersen et al. 2007). Although such pathogens are generally not lethal on their own, co-infections combined with stressful situations could reduce individual fitness and negatively affect population growth, as well as reduce juvenile survival (Forrester 1971). Inbreeding and reduced genetic variation can also interact with sublethal parasites to decrease fitness, as observed in an inbred population of Soay sheep (*Ovis aries*), where individuals with low genetic variation had more gastrointestinal parasites and lower survival rates during harsh winters than more genetically diverse sheep (Coltman et al. 1999).

Among mammals, carnivores are particularly susceptible to disease, with the highest number of species threatened by pathogens found in the canid family (Pedersen et al. 2007). Canid social behavior may explain their heightened susceptibility to pathogens as they commonly lick each other, smell and eat feces, and smell urine that may be infectious (Woodroffe et al. 2004). Other disease risk factors for wild canids include their close genetic relatedness to domestic dogs, which are globally distributed and harbor diseases easily transmissible to wild canids, their trophic position, which can expose canids to infected prey (Woodroffe et al. 2004), and their low population size. These various risk factors emphasize how disease can contribute to population declines and local extinction in canids, the best documented examples of which include: rabies in African wild dogs (*Lycaon pictus*—Gascoyne et al. 1993), gray wolves (*Canis lupus*—Chapman 1978; Ballard and Krausman 1997), and Ethiopian wolves (*C. simensis*— Sillero-Zubiri et al. 1996; Randall et al. 2004); canine parvovirus (CPV) and CDV in gray wolves (Johnson et al. 1994; Mech and Goyal 1995); and sarcoptic mange (caused

by the mite *Sarcoptes scabiei*) in arctic foxes (*Vulpes lagopus semenovi*— Goltsman et al. 1996; Ploshnitsa et al. 2011). In the United States, the red wolf (*Canis rufus*), one of the most endangered canids in the world, is emblematic of the need to evaluate and incorporate disease in canid species management.

Historically, red wolves were abundant throughout the eastern and southeastern United States, but populations were decimated in the 20th century due to habitat loss, intense predator control programs, hybridization, and disease, and the species was declared extinct in the wild by 1980 (Phillips and Parker 1988; Hinton et al. 2013). In the 1970s, the last remnant red wolves were trapped from southwestern Louisiana and southeastern Texas to start a captive breeding program. Two populations of red wolves were reintroduced, one in northeastern North Carolina (1987) and one in the Great Smoky Mountains National Park, Tennessee (1991). In 1998, Tennessee restoration efforts were discontinued due to poor pup survival associated with malnutrition and possibly parasites and CPV infections (Henry 1998). As a result, the northeastern North Carolina population, with 90–110 individuals, represents the only wild red wolf population (United States Fish and Wildlife Service 2014).

Red wolf viability had already been critically affected by disease in the remnant Louisiana-Texas population and the Smoky Mountain site, and contemporary wild red wolves in North Carolina could be vulnerable as well. North Carolina red wolves may be at risk for disease-driven declines because they persist in one small population, are inbred (Brzeski et al. 2014), and co-occur with high population density species, such as domestic dogs and coyotes (*Canis latrans*), that can be infected with the same pathogens and act as pathogen reservoirs (Eads 1948; Almberg et al. 2009). Coyotes are of particular concern because they hybridize and interact with red wolves, and although hybridization is effectively controlled by management

(Stoskopf et al. 2005; Gese et al. 2015), their frequent interaction could increase disease transmission to red wolves. Additionally, coyotes may expose red wolves to new diseases that they carry into the recovery area from surrounding regions (Hinton et al. 2012) and from elsewhere in the southeast where coyotes have been moved by humans (Hill et al. 1987).

Disease risk in the red wolf recovery area may be offset because wolves and sympatric coyotes are both opportunistically given an 8-way dog vaccination (CDV, CPV2, Adenovirus Types 1 and 2, parainfluenza, 2-Leptospirosis, and corona virus, supplied from Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri), rabies vaccination (Merial Limited, Duluth, Georgia), and flea/tick prevention when they are captured during seasonal trapping efforts. Yet, vaccines may not adequately protect red wolves because they are administered opportunistically, only a small fraction of the coyote population is vaccinated, and the efficacy of domestic dog vaccines for wild species is uncertain (Harrenstien et al. 1997; Acton et al. 2000; Acton 2008). For instance, initial vaccines are administered to wolves around 9–12 months of age, leaving younger pups exposed to infection after losing maternal antibodies around 5 months of age (Johnson et al. 1994). Another possible threat is the emergence of new vaccine-resistant viral strains, a scenario observed in Africa when a virulent new bio-type of CDV was responsible for mortality among Serengeti lions (*Panthera leo*—Roelke-Parker et al. 1996).

Potential vulnerability of red wolves to disease highlights the critical need for a systematic, focused, and informed disease monitoring and prevention plan. Evaluating pathogen loads and diversity in red wolves and sympatric coyotes, and the factors that influence disease infection are needed to inform any disease prevention plan in the recovery area. The first steps for assessing disease risk factors include an evaluation of past red wolf disease and disease occurrence in the region surrounding the North Carolina population to identify potential threats

already present on the landscape. Additionally, collecting contemporary disease data on both red wolves and sympatric coyotes will establish baseline parasite prevalence and diversity and reveal differences and similarities between the species' pathogens. To accomplish these goals, I 1) reviewed past disease occurrences in wild and captive red wolves, 2) reviewed wildlife disease literature from the southeastern United States to evaluate broadly the regional disease occurrence in mammals, and 3) collected contemporary parasite data from wild red wolves and sympatric coyotes to examine current baseline infection patterns.

METHODS

Assessment of red wolf and regional parasite literature.I reviewed existing literature on disease prevalence and risk in wild and captive red wolves by searching Web of Science for articles containing the words ["canis rufus" AND (_disease_ OR _parasit*_ OR _pathogen_)]. Additionally, I checked citations of pertinent red wolf papers to ensure that I did not miss information. I also reviewed the Red Wolf Recovery Program's records, which provide information on causes of death and necropsy results. To review literature related to infectious disease in southeastern United States wildlife populations and identify potential regional disease threats to red wolves, I searched for articles containing the words ["United States" AND south* AND (_disease_ OR _parasit*_ OR _pathogen_)] and surveyed the following journals for relevant studies: *Journal of Zoo and Wildlife Medicine*, *Journal of Wildlife Disease*, *Journal of Veterinary Medicine, American Journal of Veterinary Research, Journal of Parasitology, American Midland Naturalist*, and *Southeastern Naturalist*. I only examined articles evaluating terrestrial mammal pathogens since they are the most likely source of infections for red wolves. I also searched the Global Mammal Parasite Database, www.mammalparasites.org (Nunn and Altizer 2005) by region.

Parasite prevalence in the contemporary red wolf and coyote population. Red wolves and coyotes were trapped during the winter every year for routine management by United States Fish & Wildlife Service biologists. Canids were captured with padded leg hold traps and physically restrained for processing, during which they were weighed, aged, measured, sampled for blood, and fitted with telemetry radio-collars. I evaluated several aspects of parasite prevalence in red wolves and coyotes during this process in 2013 and 2014; I used the term parasite to include microparasites (i.e., bacteria) and macroparasites (i.e., helminths, arthropods, protozoans).

Endoparasites, which can reduce a host's physical condition and survival (Eira et al. 2006), were measured through several analyses. I collected fresh fecal samples during processing and sent them to the University of Tennessee's Veterinary Medical Center diagnostic laboratory (Knoxville, Tennessee) for sugar and zinc fecal floats to assess species prevalence and individual infection levels. Infection levels were based on the number of eggs, cysts, or oocysts detected on fecal slides surveyed at 10× magnification across 12 transects, where none = no eggs, cysts, or oocysts detected; low = 1–12 eggs, cysts, or oocysts; intermediate ≥ 12, but eggs, cysts, or oocysts not present on every transect; heavy ≥ eggs, cysts, or oocysts on every transect. I tested for canine heartworm (*Dirofilaria immitis*) infections with SNAP Heartworm RT Tests (IDEXX Laboratories, Westbrook, Maine) in 2013 and SNAP 4Dx Tests (IDEXX Laboratories) in 2014. I tested for CPV in 2013 with SNAP Parvo Tests (IDEXX Laboratories), but as no active infections were detected, I did not test for CPV in 2014. I also tested for tick-borne illnesses with SNAP 4DX Tests, which provide a negative or positive for bacteria causing Lyme disease (*Borrelia burgdorferi*), and for *Ehrlichia* spp. (*E. canis* or *E. ewingii*), and *Anaplasma* spp. (*A. platys* or *A. phagocytophilum*).

I evaluated ectoparasite infestations for each canid by inspecting the neck, ears, perianal area, and axillae. I removed ectoparasites by hand or with a flea comb, storing them in 70% ethanol; combs were sterilized between canids. Ectoparasites were grouped by order and counted to establish an ectoparasite load for each captured canid; loads were defined as few (< 5), intermediate (5–100), and heavy (> 100). All research on live canids followed the guidelines of the American Society of Mammalogists (Sikes et al. 2011) and was approved by the Louisiana State University AgCenter Animal Care and Use Committee (protocol # A2013-16).

Statistical methods.I compared endoparasite communities (including heartworm) in red wolves and coyotes with rarefaction estimates of species richness using the program EstimateS version 9.1.0 (Colwell 2013). The sample-based, rarefaction method estimates the expected number of parasite species represented among red wolves and coyotes, given the observed samples to generate predicted estimates of parasite richness. I also extrapolated the rarefaction curve to a sample size of 50 canid individuals to evaluate how endoparasites species richness varied between red wolves and coyotes with equal and larger sample sizes. I based significant differences between red wolf and coyote rarefaction estimates on nonoverlapping 95% *CI*s generated through bootstrapping routinesin EstimateS, which is a conservative estimate of significance (Colwell et al. 2012).

I assessed factors influencing parasite infections with generalized linear mixed effect models (GLMMs) using the R package lme4 (Bates and Maechler 2010) and with cumulative link mixed models (CLMMs) using the R package ordinal (Christensen 2012). Explanatory variables for each model included age class, sex, species, and year collected with a random effect of region captured (coyotes) or pack (wolves). I included random effects to control for nonindependence between individuals from the same pack or trapping region. I defined age

classes as pup (less than 12 months old), juvenile (greater than 12 months but under 2 years), and adult (greater than 2 years); I determined age by date of birth for wolves and based estimated ages on tooth wear (Gier 1975) and sexual maturity for coyotes. I ran 12 a priori candidate model sets, including a null and global model (Tables C1–C8), separately for each of the following response variables: endoparasite counts (tally of infectious species, weighted by infection level), heartworm presence, ectoparasite loads, and any other pathogenic parasite (either individual endoparasites species or tick-borne bacteria) with an observed infection rate above 10%. I evaluated the probability of specific endoparasites species, heartworm, and tick-borne bacteria using GLMMs with a logit-link function and binomial error distribution; models with ectoparasite loads were evaluated using CLMMs with a log-link function. I assessed endoparasite counts using GLMMs with a log-link function and Poisson distribution. All models were ranked with AICc and AICc weight (*wi*—Burnham and Anderson 2002) and validated by examining residuals and fitted values as suggested by Zuur et al. (2009). I averaged models within Δ2 AICc of the top model using the naturalaverage method (Burnham and Anderson 2002) in R package MuMIn (Bartoń 2009); I also used analysis of variance to evaluate if additional variables significantly improved model fit. Given that adult heartworm prevalence was high, I evaluated if adult red wolves were more likely to have heartworm than adult coyotes with Fisher's exact test; I were unable to test this with GLMMs given small sample sizes (adult red wolves tested for heartworm = 13, adult coyotes tested for heartworm = 10).

RESULTS

Red wolf literature. The last free ranging red wolves in the historic Louisiana and Texas populations had high infection rates of hookworm (Riley and McBride 1972; Carley 1975; Custer and Pence 1981a), heartworm (*D. immitis*—Riley and McBride 1972; Carley 1975;

Custer and Pence 1981b), and sarcoptic mange (*S. scabiei*—Riley and McBride 1972; Carley 1975; Pence et al. 1981). All 3 parasites were considered limiting factors to red wolf survival and may have affected morbidity and mortality significantly (Riley and McBride 1972; Carley 1975; Custer and Pence 1981b). Hookworm infections were especially high in pups and juveniles and may have been a leading cause of juvenile mortality (Custer and Pence 1981a). The severity of heartworm infections increased with age (Custer and Pence 1981b), resulting in pathological responses such as enlarged and deformed hearts, and increasing stressinduced mortality that healthy wolves would likely have survived (Riley and McBride 1972; Carley 1975). Sarcoptic mangewas the most serious ectoparasite; infections were so numerous that by the 1970s, 90% of observed red wolves were at least partially devoid of hair (Riley and McBride 1972). Other detected parasites included tapeworm (*Taenia* sp.), demodectic mange mites (*Demodex* sp.), spiny headed worms (class Archiacanthocephala), flatworms (*Heterobilharzia americana*), several species of ticks (*Amblyomma* sp., *Ixodes scapularis*), and 1 louse (*Trichodectes canis*—Riley and McBride 1972; Custer and Pence 1981a; Pence et al. 1981).

Heartworm, endoparasite, and ectoparasite prevalence were evaluated in several of the first reintroduced wild wolves in North Carolina, as well as in captive wolves housed at the initial North Carolina release site (Phillips and Scheck 1991). No captive red wolves had heartworm, and only 1 of 7 tested wild wolves was heartworm positive. Wild adult wolves, however, had been regularly treated with ivermectin, a heartworm prophylactic, prior to release. Captive red wolves had fewer endoparasites (48% infected) than wild wolves (67% infected), but both were infected with several different intestinal parasites including hookworms (both wild and captive wolves), ascarids (more common in captive wolves and only found in pups), whipworms (wild only), and tape-worms (both wild and captive wolves—Phillips and Scheck 1991). Phillips

and Scheck (1991) suggested that hookworm was the only parasite occurring at high enough frequencies to be of concern to red wolf health. Three tick species, American dog tick (*Dermacentor variabilis*), lone star tick (*Amblyomma americanum*), and black legged tick (*I. scapularis*), were detected on wild and captive wolves (Phillips and Scheck 1991). Since reintroductions, several tick related illnesses have been detected in wild wolves. Tick paralysis may have occurred in a female red wolf from North Carolina and was positively observed in 1 male, who recovered fully once ticks were removed (Beyer and Grossman 1997). Several red wolves housed at the Great Smoky Mountains National Park were serologically positive for the bacteria causing Lyme disease (*B. burgdorferi*); one positive wolf also exhibited *B. burgdorferi* clinical symptoms, including decreased appetite, weight loss, and carpal lesions (Penrose et al. 2000).

Acton (2008) evaluated CPV2 and CDV prevalence in northeastern North Carolina carnivores, including red wolves, and assessed vaccine efficacy. Based on samples collected from 2000 to 2006, red wolves and coyotes were naturally exposed to both CPV2 and CDV, but North Carolina canid titers were lower than those for other wild canid populations (Acton 2008). CDV vaccines appeared to elicit 100% seroconversion, or the development of detectable vaccine antibodies, but CPV2 vaccines did not reliably elicit seroconversion (Acton 2008). This is similar to results reported by Harrenstien et al. (1997), where red wolf response to CPV2 vaccines was minimal. Based on seroprevalence, poor vaccine efficacy, and neonatal antibody assays, Acton (2008) suggested that CPV2 may contribute to juvenile mortality in wild red wolves. A recent study by Anderson et al. (2014) found 100% and 96.9% of captive wolves had positive CPV and CDV vaccine titers, respectively, 3 years after vaccination, but this was after a

full juvenile vaccination series and a 1 year booster, which wild canids usually do not receive. Seroconversion for canine adenovirus was sporadic (Anderson et al. 2014).

Several additional studies document rare medical conditions in captive red wolves, such as bilateral idiopathic dry eye, pyometra, and paten ductus venosus (Day et al. 1992; Neiffer et al. 1999; Kearns et al. 2000; Crissey et al. 2001; Larsen et al. 2002; Acton et al. 2006; Anderson and Wolf 2013). A comprehensive necropsy survey in the captive breeding program documented several causes of death, including neonatal parasitism, cardiovascular and gastrointestinal problems, and possibly one CPV mortality, but chronic infectious diseases did not appear to be a widespread problem (Acton et al. 2000).

Records from the Red Wolf Recovery Program indicated that mange contributed to the death of 18 red wolves in the wild North Carolina population since 1993, and in 46 additional documented cases of mange, wolves were treated and released; both sarcoptic and demodectic mange were identified. Heartworms were regularly reported and have been confirmed as the cause of mortality for 9 wolves. One wolf died due to complications with heartworm treatment; Red Wolf Recovery Program biologists no longer attempt to treat heartworm infections in wild wolves. One wolf died due to CPV.

Disease review in southeastern United States.I reviewed 185 references that reported wildlife pathogens in the southeastern United States. The most reported, and probably the most tested, viral pathogens were CPV, CDV, rabies, canine adenovirus, and equine encephalitis virus, all of which are pathogenic in canids (Table C9). Endoparasites, which include organisms such as Cestodes, Nematodes, Protozoa, and Trematodes, were the most commonly evaluated parasite and were widespread across different host species throughout the southeastern states (Table C10). Given their prevalence and pathology, several endoparasite species (currently absent in red

wolves) may be of particular concern: *Babasia* spp., causing lethargy and neurologic problems (Birkenheuer 2014); *Hepatozoon* spp., causing fever, lameness, lethargy, and skeletal lesions (Vincent-Johnson 2014); *Toxocara* spp., which was detected in 1 North Carolina coyote and can cause lethargy and intestinal distress; *Toxoplasma gondii*, causing organ lesions (Lappin 2014); and, *Trypanosoma cruzi*, causing lethargy, loss of appetite, and sudden death (Barr et al. 2014; Table C10; see also Table C11 for disease occurrence in North American canids).

There were several tick-borne bacterial pathogens with high incidence rates in the Southeast including *Ehrlichia* spp., *Borrelia burgdorferi* (bacteria causing Lyme disease) and *Leptospira* spp. (bacteria causing Leptospirosis; Table C12). *Leptospira* spp., although included in the administered 8-way vaccine and never detected in red wolves, may be a future concern given it is epizootic in domestic dogs and causes symptoms such as fever, lethargy, reluctance to move, anorexia, and respiratory difficulty (Sykes 2014).

Contemporary red wolf and coyote parasite prevalence. During the winters of 2013 and 2014, 37 red wolves, 51 coyotes, and 3 hybrids (included with coyotes in my analyses) were trapped and examined. One red wolf and 1 coyote were captured in both years; I only analyzed data from their first complete sampling. Fecal parasites were analyzed for 49 individuals, 69 were tested for heartworm, 56 were tested for tick-borne pathogens, and 91 canids were evaluated for ectoparasite loads. Coyotes harbored more endoparasite species than did red wolves based on rarefaction curves but 95% *CI*s overlapped between the species (Fig. 4.1). The species accumulation curves showed that parasite richness of red wolves appeared to plateau while coyotes were projected to accumulate more parasites (Fig. 4.1). Of the 20 different fecal pathogen species detected, 6 are considered nonpathogenic to canids or were possibly incidental ingestions, e.g., mites (Table 4.1).

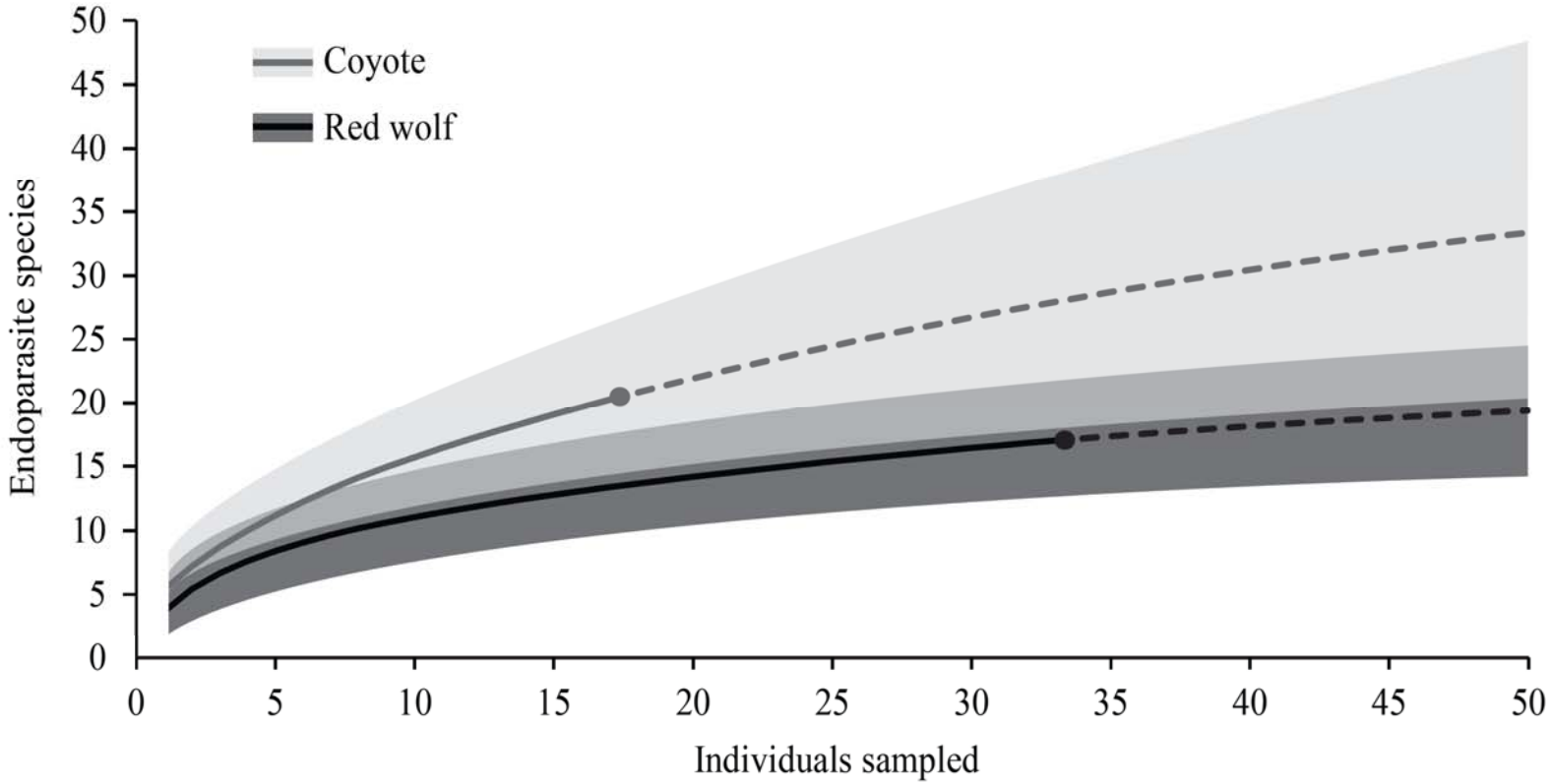

Figure 4.1. Estimated number of endoparasites in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina based on rarefaction (solid lines) and extrapolation (hashed lines). The shaded regions denote 95% confidence limits. Sample sizes, indicated by the solid circles, varied by species (red wolf =33, coyote =17).

Table 4.1. Endoparasites detected in endangered wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina 2013 and 2014.

Parasite	Red wolf (n=33)	Coyote (n=17)	Prevalence
Helminths			
Ancylostoma spp. *(canimum)*[1,2]	31	16	94%
Capillaria spp.[1]	2	6	16%
Eucoleus aerophilus	1	0	2%
Eucoleus boehmi	1	1	4%
Filaroides osleri	0	1	2%
Hymenolepis diminuta	2	2	8%
Physaloptera spp.[1]	0	1	2%
Spirometra	6	2	16%
Taeniid type eggs[1,2,3]	5	1	12%
Toxocara canis	0	1	2%
Trichuris vulpis[1,2]	1	3	8%
Uncinaria stenocephala	11	5	32%
Protozoa			
Cystoisopora canis	1	1	4%
Cystoispora ohioensis	6	1	14%
Neospora/ Hammondia	1	1	4%
Sarcocystis spp.	24	12	72%
Arthropoda			
Demodex spp.	1	3	8%
Louse spp[4]	0	1	2%
Mite spp[4]	11	5	32%
Coccidia			
Eimeria spp.	2	1	6%

[1]Endoparasite species previously detected in remnant LA and TX red wolf population.
[2]Endoparasite species previously detected in current NC red wolf population.
[3] *Taenia* spp. and *Echinoccus* spp. eggs are indistinguishable and can only be categorized by egg.
[4] Mite and louse species may be incidental and nonpathogenic.

The most prevalent fecal pathogens, with detection rates over 10%, included *Ancylostoma* spp., *Uncinaria stenocephala*, *Capillaria* spp., *Cystoisospora ohioensis*, *Spirometra*, *Sarcocystis* spp., and *Taeniid* type eggs (*Taenia* spp. and *Echinococcus* spp. eggs are indistinguishable and can only be categorized by egg type). *Ancylostoma* spp. was the most common endoparasite and was detected in 94% of individuals. GLMM model results suggest young canids had more endoparasites (Fig. 4.2; Table C1). Year of sampling was also within the

top Δ2 AICc models but *CI*s overlapped zero (Table 4.2). Most GLMM models with individual endoparasite species either encompassed the null model within the top Δ2 AICc models or did not converge (Table C2–C4), except for *U. stenocephala* models, where canids captured in 2014 were less likely to have *U. stenocephala* infections (Table 4.2; Table C5).

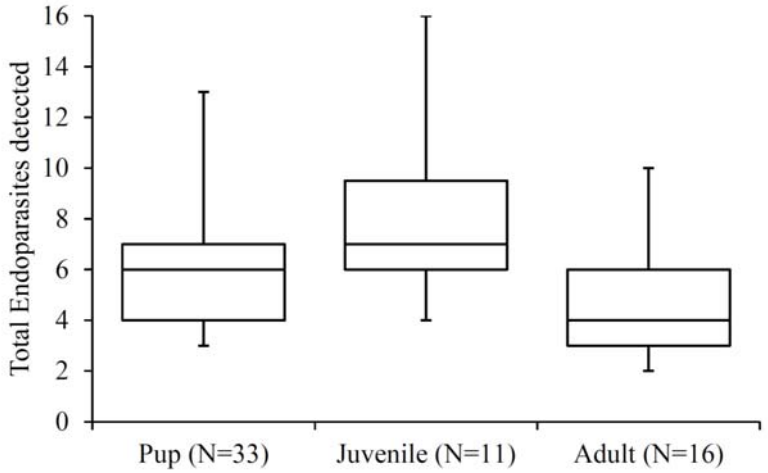

Figure 4.2. Box-and-whisker plot comparing total endoparasites detected in different age classes of endangered wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina. The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation in each age class. Pups (under 12 months) and juveniles (between 12 and 24 months) were more likely than adults (over 24 months) to have higher endoparasite loads.

Heartworm prevalence was high with a 45% infection rate (Fig. 4.3), and based on the top GLMM model, age class significantly influenced probability of infection where adults were more likely to have heartworm (Table 4.2; Table C6). Year of collection was included within the top Δ2 AICc models, but *CI*s overlapped 0 (Table 4.2). Species and sex did not affect the probability of heartworm infection significantly. Adult red wolves appeared to be more susceptible to heartworm than adult coyotes (P = 0.02; Fisher's exact test).

Table 4.2. Parameter estimates (β), adjusted standard error (SE), and 95% confidence limits (CL) of variables in the final averaged models evaluating infection probability of total endoparasites detected, heartworm, and ectoparasites in endangered wild red wolf (*Canis rufus*) and sympatric coyotes (*Canis latrans*).

Dependent	Explanatory	β	SE	Z-	*P*	CL	CL
Endoparasite	Age class (adult)	1.58	0.14	11.39	0.00	1.31	1.85
	Age class	0.54	0.17	3.19	0.00	0.21	0.87
	Age class (pup)	0.33	0.16	2.01	0.04	0.01	0.65
	Year (2014)	-	0.14	0.86	0.39	-0.40	0.16
Uncinaria	Year (2014)	-	0.81	2.94	0.00	-3.95	-0.79
	Age class (adult)	0.86	0.81	1.07	0.29	-0.72	2.45
	Age class	-	0.65	0.36	0.72	-3.12	0.87
	Age class (pup)	-	0.75	0.43	0.66	-3.29	0.20
	Sex (M)	-	0.47	0.32	0.75	-2.33	0.80
	Species (Red	-	0.54	0.32	0.75	-2.68	0.94
Heartworm	Age class (adult)	2.19	0.78	2.81	0.00	0.66	3.71
	Age class	-	0.85	3.23	0.00	-4.43	-1.08
	Age class (pup)	-	0.93	3.79	0.00	-5.33	-1.69
	Year (2014)	-	0.73	1.94	0.05	-2.85	0.01
Ehrlichia[1]	Age class (adult)	0.61	0.61	1.00	0.32	-0.58	1.79
	Age class	-	0.79	0.73	0.46	-2.14	0.97
	Age class (pup)	-	0.77	2.42	0.02	-3.37	-0.36
	Year (2014)	-	0.65	0.87	0.39	-1.83	0.71
	Species (Red	0.54	0.77	0.70	0.48	-0.97	2.06
Ectoparasite load	Year (2014)	2.27	0.76	2.98	0.00	0.8	3.8
	Species (Red	0.72	0.65	1.10	0.27	-0.6	2.0

[1]Null model within Δ2 AIC_c of the top model.

The occurrence of tick-borne diseases varied. Five canids tested positive for Lyme disease: 2 adult male red wolves and 3 coyotes (2 juveniles, 1 pup). One adult male red wolf that tested positive for Lyme disease was in poor condition when trapped and showed symptoms of mange. Due to health concerns, he was tested at a local vet where he was found positive for Lyme disease, *Ehrlichia* spp., and Rocky Mountain spotted fever (*Rickettsia rickettsii*—A. B. Beyer, U.S. Fish & Wildlife Service, April 2013). This same male was recaptured in 2014 in poor condition and was found to be positive for Rocky Mountain spotted fever but not Lyme disease; he was held and re-treated. All of the positive Lyme disease canids were trapped in 2013

except for the one positive coyote pup, which was trapped in 2014 (Table 4.3). There were no conclusive *Anaplasma* spp.- positive canids, although one male coyote trapped in 2013 had a faint, inconclusive positive SNAP test result. *Ehrlichia* spp. were common, with a 45% infection rate. The top GLMM model indicated older canids were more likely to have *Ehrlichia* spp. infections (Fig. 4.4). Sex, species ID, and year had little influence on the probability of infection but the null model was within Δ2 AICc of the top model (Table 4.2; Table C7).

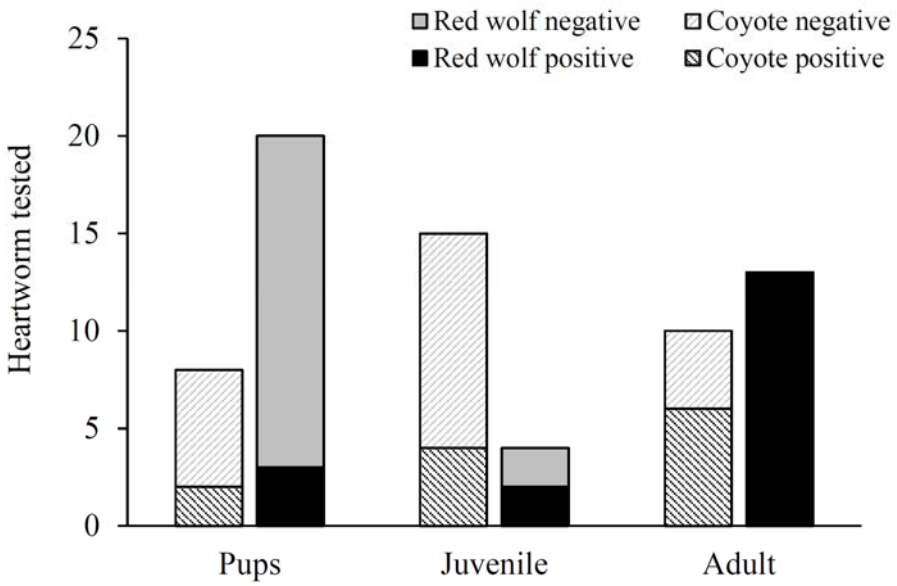

Figure 4.3. Heartworm (*Dirofilaria immitis*) prevalence among wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in North Carolina. Adults (older than 2 years) were more likely than pups (under 12 months) or juveniles (between 12 and 24 months) to be heartworm positive; adult red wolves may also be more susceptible than adult coyotes to heartworm.

The most common ectoparasites were ticks and biting lice. Individuals were more likely to have higher ectoparasite loads in 2014 than 2013 (Fig. 4.5), but age class and sex had no effect (Table 4.2; Table C8). Species ID was within the top Δ2 AICc models where red wolves were more likely to have higher parasite loads than coyotes, but *CI*s overlapped 0 (Table 4.2).

Table 4.3. Number of tick-borne pathogens (Lyme disease, *Anaplasma* spp., and *Ehrlichia* spp.) detected in endangered wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina, 2013 and 2014. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

	Red Wolves						Coyotes					
	Lyme		*Anaplasma*		*Ehrlichia*		Lyme		*Anaplasma*		*Ehrlichia*	
Age	-	+	-	+	-	+	-	+	-	+	-	+
Pups	17	0	17	0	14	3	4	1	5	1[1]	3	2
Juvenile	4	0	4	0	2	2	10	2	11	0	7	5
Adult	9	2	10	0	2	8	10	0	10	0	5	5
Total	30	2	31	0	18	13	24	3	26	1[1]	15	12

[1]Inconclusive.

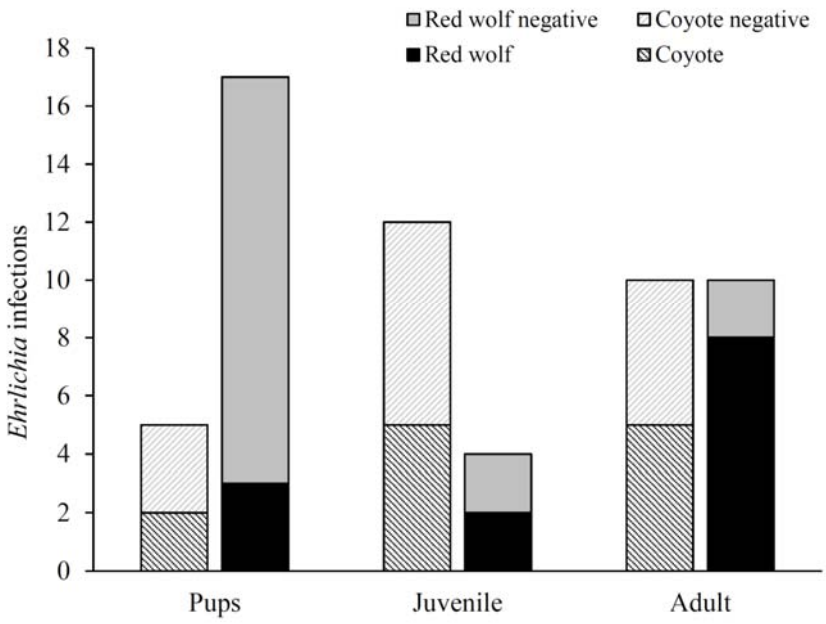

Figure 4.4. *Ehrilichia* spp. prevalence among endangered wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina; white bars represent negative test results. Marginal evidence suggests adults (older than 2 years) were more likely than pups (under 12 months) or juveniles (between 12 and 24 months) to be *Ehrilichia* spp. positive.

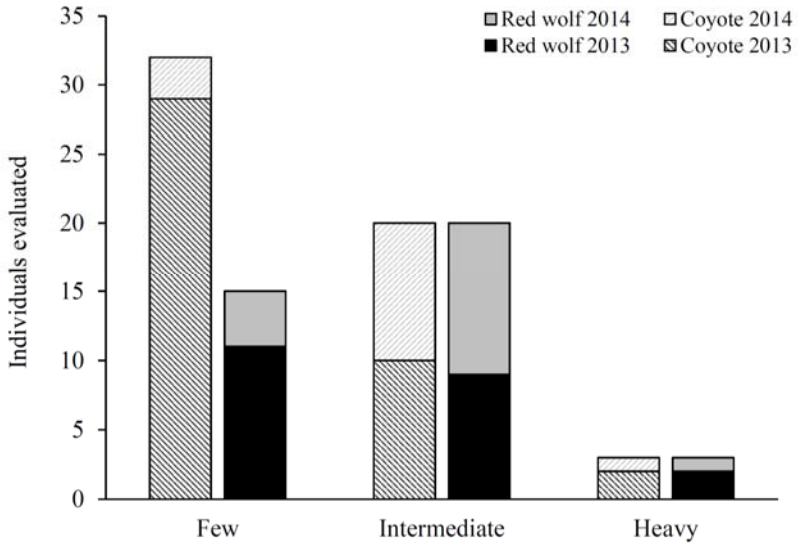

Figure 4.5. Ectoparasite loads detected on endangered wild red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) in northeastern North Carolina. The likelihood of having heavier ectoparasite loads was greater in 2014 than 2015.

DISCUSSION

I assessed past red wolf disease occurrence, regional disease threats (Table C9–C13), and collected baseline parasite data on endangered red wolves (Tables 4.1 and 4.3) to inform a monitoring plan aimed at preventing disease-mediated population declines in red wolves. My results highlight several possible pathogen threats to contemporary wild red wolves: (i) coyotes, which may act as a source or reservoir for disease, and (ii) several regional diseases that are prevalent on the landscape and could be detrimental to the small red wolf population.

Coyotes may be a disease threat because their endoparasites community has greater species richness than red wolves and it is projected to increase with more intensive sampling (Fig. 4.1). Interactions between coyotes and red wolves may facilitate disease transmission between the species, leading to the introduction of new pathogens to the red wolf population. This could affect long-term population recovery because small, endangered populations like red wolves are likely to be immunologically naïve and lack the genetic variation necessary to combat

new diseases (Spielman et al. 2004). Interestingly, coyotes and red wolves did not significantly differ in their probability of infection in any of the parasites I evaluated (Table 4.2), with the exception that adult red wolves were more susceptible to heartworm than coyotes and twice as many coyotes tested positive for the bacteria causing Lyme disease. Perhaps differences in red wolf and coyote diet, foraging behavior, or habitat preference cause differential exposure to the heartworm and Lyme disease vectors: mosquitoes and *Ixodes* ticks, respectively. Long-term temporal data would help determine with more certainty if coyotes act as a disease reservoir and inform the dynamics of disease transmission between the species.

Mange was identified as an important parasite to monitor in the red wolf recovery area and the southeastern region. Mange had caused mortalities in coyotes and foxes regionally (Table C10) and has already impacted red wolves, killing at least 18 wolves in the North Carolina population. Mange epizootics likely do not have long-term demographic effects for common species like coyotes or foxes but can be devastating to small populations such as red wolves because the loss of just a few individuals can reduce population growth (Pence and Ueckermann 2002) or even lead to local extinction (Henriksen et al. 1993; Ploshnitsa et al. 2011). Treatment of mange is difficult in wild animals because it requires capturing and administering ivermectin to both infected individuals and those they contacted (Bornstein et al. 2001) but would be warranted for red wolves if a mange epizootic occurred since there have been 46 cases of mange infections successfully treated in wild red wolves.

The most virulent regional disease threats detected were viral infections such as CPV, CDV, and rabies (Dobson and Foufopoulos 2001; Pedersen et al. 2007; Smith et al. 2009), which were widespread in southeastern wildlife populations (Table C9; see also Table C13 for disease-driven declines in threatened species). Although currently these viruses do not appear to be

epizootic within the southeast region (although see Dyer et al. 2013; Table C9), the red wolf population may be at risk. Wild red wolves and sympatric coyotes have been exposed to both CPV and CDV in North Carolina (Acton 2008), where at least one red wolf death was attributed to CPV. Red wolves can mount a positive serological response to CPV and CDV vaccines, but the efficacy of opportunistic vaccinations in the wild population is not well established (Acton 2008; Anderson et al. 2014).

Another consideration is the long-term effects of prophylactic vaccination and medical treatments. Vaccines and other interventions such as ivermectin for mange could have negative evolutionary consequences in wild populations because selection pressures for immunity may be weakened with continued treatment. Opportunistic vaccines and treatments that do not provide life-long immunity could also result in multiple individuals becoming susceptible to disease simultaneously, increasing the risk of an epizootic (Woodroffe 1999). The potential drawbacks of vaccines and medical intervention need to be considered by managers and the risk of infection found sufficient to justify intensive prevention efforts. For red wolves, the very real risk of extinction due to their extremely small population size outweighs the potential negative effects of intervention, especially for virulent viral pathogens such as rabies and treatable conditions like mange. As the red wolf population increases and additional wild population are established, vaccinations and intensive treatment may no longer be necessary.

The most prevalent parasites detected in red wolves during my 2013–2014 sampling were hookworm (Ancylostoma spp.) and heartworm (D. immitis), both of which were widespread throughout southeastern wildlife as well; positive infection rates were 94% and 45%, respectively. Hookworm increased pup mortality in the remnant Louisiana and Texas population (Custer and Pence 1981a) and remains a management concern due to its current prevalence rate

and high loads in young wolves (Fig. 4.2). High heartworm prevalence may be a more immediate threat because heartworm infections have caused the death of at least nine red wolves and adults may be especially susceptible to them (Fig. 4.3). Compounding the negative effects of hookworm and heartworm is that wild red wolves are inbred (Brzeski et al. 2014), which may cause them to suffer more from co-infections or stressful conditions than an outbred population, like coyotes (Coltman et al. 1999; Spielman et al. 2004). Management efforts, such as cross fostering captive born pups into wild litters, can help mitigate the deleterious effects of inbreeding (Brzeski et al. 2014), but continued monitoring of endoparasites and more rigorous demographic modeling of the impact of heartworm related deaths will be useful for future disease prevention.

The detection of tick-borne diseases is an additional risk factor for red wolves and wildlife in general because the expansion of vector-borne diseases have been associated with climate change (Sutherst 1998; Patz et al. 2008). For instance, climate and landscape changes have facilitated the spread of the bacteria causing Lyme disease, *B. burgdorferi*, and based on climate models, Lyme disease is expected to continue to expand northward (Ostfeld et al. 1996; Ogden et al. 2006). The presence of Lyme disease, *Ehrlichia* spp., and Rocky Mountain spotted fever in red wolves and coyotes serves as a benchmark for detecting the emergence of additional vector-borne pathogens in North Carolina.

Currently, disease may not be the primary threat to red wolf recovery given that there were no major disease outbreaks or frequent red wolf mortality events directly caused by disease. This may be due in part to vaccines and medical interventions, or wild red wolves may not have been exposed to extremely virulent pathogens. But the prevalence rate of parasites in the red wolf and sympatric coyote populations as well as several regional trends reveal substantial

concerns. In a critically endangered population such as wild red wolves, every wolf is important for species persistence and pathogens that reduce fitness, result in occasional deaths, or even moderately affect population growth could contribute to extinction (Woodroffe 1999). To mitigate disease-driven declines, endangered species programs such as the Red Wolf Recovery Program must incorporate disease monitoring and prevention plans to ensure long-term recovery, the first steps of which I presented here.

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CHAPTER 5: IMMUNOGENETIC VARIATION AND DISEASE SUSCEPTIBILITY IN ENDANGERED RED WOLVES (*Canis rufus*) AND SYMPATRIC COYOTES (*Canis latrans*)

INTRODUCTION

Organisms' basic biology and life-history strategies can be shaped by efforts to avoid, detect, and defeat parasite infections (Harvell 2004, Sommer 2005). In addition to observable consequences of parasites on biological systems, such as near extinctions and population crashes of host populations (Woodroffe 1999; de Castro and Bolker 2004), the antagonistic dynamics between parasites and hosts are often measureable at the molecular level. Alleles that provide disease resistance will be selected for and maintained at the population level through parasite-mediated frequency dependent selection (Decaestecker et al. 2007) or heterozygous advantage (Froeschke and Sommer 2005). Alternatively, alleles which confer a strong immunological advantage could be driven to fixation by directional selection (Woolhouse et al. 2002). Despite sustained interest in measuring a host's molecular response to disease for scientific and conservation purposes, the outcome of parasite-mediated selection is still not well understood in free living wildlife communities (Spurgin and Richardson 2010).

The paucity of studies from free-living wildlife stems from, in part, the complexity of vertebrate immune systems and difficulty in detecting direct associations between immune genes and parasite susceptibility (Boughton et al. 2011, Demas et al. 2011). There are two major immune mechanisms that can be examined in vertebrates: the innate and the adaptive immune response (Demas et al. 2011). The innate branch of the immune system activates the adaptive response and is rapid, non-specific, and the first line of defense against invading pathogens (Demas et al. 2011). Important genes for innate immunity include toll-like receptor (TLR) genes. TLR genes encode pattern recognition receptors that detect pathogen-associated molecular

patterns (PAMPs) not present in the host, such as bacterial lipoprotein and flagellin (Iwasaki and Medzhitov 2004). PAMP recognition activates transcription factors that induce expression of interferons, proteins that initiate an immune response cascade (Alcaide and Edwards 2011, Iwasaki and Medzhitov 2011). Researchers have identified associations between TLR gene polymorphism and disease susceptibility in various species (Leveque et al. 2003, Schroder and Schumann 2005, Misch and Hawn 2008, Kathrani et al. 2010); however, these have not been extensively studied in wild populations. Initial research on wild populations demonstrates TLR gene polymorphisms can confer resistance to *Borrelia afzelii* in wild bank voles (*Myodes glareolus*; Tschirren et al. 2013) and are associated with ectoparasite burden in wild water voles (*Arvicola amphibius*; Gavan et al. 2015). However, other work has failed to find associations between *Toxoplasma gondii* and TLR polymorphisms in wild woodmice (*Apodemus sylvaticus*; Morger et al. 2014).

The adaptive immune response is a slower, pathogen-specific response that often provides lifetime protection against reinfection from the same parasite (Boughton et al. 2011, Demas et al. 2011). Adaptive immune capabilities can be evaluated by examining major histocompatibility complex (MHC) genes (Loisel et al. 2007, Setchell et al. 2010). The MHC is a highly variable gene complex that encodes proteins to detect and present foreign bodies to T-cells. Thus, an individual's ability to recognize a variety of pathogens is partially dependent on the number and diversity of MHC alleles expressed (Grob et al. 1998). Correlations between MHC alleles, haplotypes, or heterozygosity and pathogen resistance have been shown for a number of species (reviewed in Sommer 2005, Lenz et al. 2009, Oliver et al. 2009). Many previous studies have focused on variation at a single immune gene complex, most notably MHC, but evaluating both innate and adaptive immune genes provides a more complete picture

of immunogenetic variation and parasite mediated selection (Acevedo-Whitehouse and Cunningham 2006).

Elucidating how pathogen mediated selection shapes genetic variation has broad evolutionary importance, but there is also significant conservation value in understanding how disease susceptibility is influenced by immunogenetic variation. This is especially true in small populations of endangered species where inbreeding increases homozygosity and drift erodes genetic variation, potentially increasing risk of disease mediated population declines (Spielman et al. 2004, Frankham et al. 2010). Endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*) provide an opportunity to evaluate associations between immunogenetic variation and parasite susceptibility in sympatric wild species that have different population sizes and may have differential selection pressures. Red wolves went through an extreme bottleneck and currently persist in only one small, inbred, wild population in northeastern North Carolina (Hinton et al. 2013, Brzeski et al. 2014), whereas coyotes are an abundant and widespread species. Red wolves and coyotes have similar pathogen communities but coyotes may have higher parasite diversity (Brzeski et al. 2015) and are likely exposed to more generalist parasites than red wolves because of their larger geographic range. The disparate demography and range size of red wolves and coyotes could lead to differential immunogenetic outcomes, where coyotes likely have higher immunogenetic variation and may also be subjected to stronger or more variable parasite mediated selection given they are exposed to more heterogeneous conditions than isolated red wolves (Lazzaro and Little 2009). Red wolves, in contrast, likely have low immune gene variation as a consequence of a small population size, therefore immune gene evolution may be more reliant on genetic drift, and not display similar signatures of selection.

The red wolf-coyote dynamic in North Carolina provides an opportunity to assess immunogenetic variation and parasite susceptibility in a small, inbred wild population in conjunction with a sympatric outbred population. My objective was to assess immunogenetic variation and detect associations between parasite measures and immune genes to better understand both disease susceptibility and mechanisms maintaining genetic diversity in both species. To accomplish this I sequenced TLR and MHC genes, and evaluated immunocompetence with individual bacterial killing capacity and white:red blood cell ratios, and disease prevalence by estimating parasite loads in red wolves and sympatric coyotes.

METHODS

Red wolves were extirpated from the wild in the late 1970s, but through a captive breeding program, wolves were reintroduced into northeastern North Carolina starting in 1987 (Hinton et al. 2013). The current wild population is genetically represented by 12 of the 14 red wolf captive breeding founders. The North Carolina recovery area once encompassed 1.7 million acres throughout 5 counties in northeastern North Carolina (Dare, Tyrrell, Hyde, Beaufort, and Washington counties; USFWS 2014). Originally the recovery area had no coyotes, but due to coyote range expansion eastward, hybridization was documented in 1993. However, hybridization has been well managed and minimal introgression appears to have occurred (Bohling and Waits 2015). USFWS (United States Fish and Wildlife Service) Red Wolf Recovery Program biologists trap red wolves and sympatric coyotes from November to May; during this process I collected pathogen data and blood for DNA analysis. Additionally, red wolves and coyotes were both opportunistically given an 8-way dog vaccination (CDV, CPV2, Adenovirus Types 1 and 2, parainfluenza, 2-Leptospirosis, and corona virus, supplied from Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri), rabies vaccination (Merial

Limited, Duluth, Georgia), and flea/tick prevention when they were captured during seasonal trapping efforts. However, these efforts were sporadic and only a small fraction of the coyote population was treated.

TLR and MHC sequencing. In mammals, there are 10-15 TLR gene families, of which TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 have been characterized or sequenced in canids (Bozzocchi et al. 2005, Hashimoto et al. 2005, Ishii et al. 2006, Kathrani et al. 2010, Huber et al. 2011). I sequenced TLR1, 4, 5, and 6, which are representative of 3 subfamilies: subfamily TLR1 (including TLR1 and TLR6), subfamily TLR4 (TLR4), and subfamily TLR5 (TLR5). These four TLR genes recognize non-viral ligands, have been found to be under positive selection in various species, and are important in recognizing bacteria lipoproteins (TLR1 and TLR6), lipopolysaccharides (TLR4), and flagellin (TLR5) on the cell surface (Iwasaki and Medzhitov 2004, Areal et al. 2011). Primers were available for full length TLR 1, 4, and 6 genes (Table D1). I designed internal TLR5 primers which amplified a variable portion of the TLR5 exon with NCBI Primer-BLAST (Ye et al. 2012), using available canine TLR5 sequences. I also redesigned internal forward primers for TLR1 and TLR4 exon 3 genes to target detected SNPs (Table D1).

In the MHC, I sequenced coding exon 2 in three dog leukocyte antigen (DLA) class II genes DRB1, DQA1, and DQB1. MHC class II primers have been previously developed and extensively used in other canid species (Seddon et al. 2002, Kennedy et al. 2007, Wilbe et al. 2009, Kennedy et al. 2011), as well as in captive red wolves, where Hedrick et al. (2000, 2002) examined the class II DRB gene. They found red wolves were relatively depauperate at the DRB gene but that there was strong evidence for balancing selection (Hedrick et al. 2000, 2002). I used primers as described in Wagner et al. (1996, 1999), and Kennedy et al. (2007; Table D1).

I extracted genomic DNA from red wolf and coyote blood samples stored in Queen's buffer (Seutin et al. 1991) with QIAGEN® DNeasy blood and tissue kits (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Polymerase chain reactions (PCR) included 0.25 ng DNA, 0.25 U Taq DNA Polymerase (New England Biolabs),1x Standard buffer (New England Biolabs), 1.5 μM $MgCl_2$, 200 μM dNTPs (QIAGEN), 0.05 μM primer and nanopure water for a final volume of 25 μl. PCR conditions varied by primer pair (Table D1). All PCR product was sent to Beckman Coulter Genomics (Danvers, MA) for bi-directional Sanger sequencing. I cloned and sequenced a subset of samples at MHC genes to confirm the presence of unique or new alleles (Table D2). For cloning, I followed the same conditions as for sequencing but ran reactions in a 50 μl volume and sent PCR product to MClab (San Francisco, CA) for cloning and sequence verification. All sequences were edited, aligned, and compared with SEQUENCHER v5.1 (Gene Codes Corporation, Ann Arbor, MI USA).

For heterozygous TLR sequences, I resolved individual haplotypes using PHASE as implemented in DnaSP v5 (Librado and Rozas 2009) using default parameters with 100 iterations, one thinning interval, and 100 burn-in iterations. MHC haplotypes were resolved with a stepwise, subtractive approach as described previously (Kennedy et al. 2002a, b). Briefly, I first identified haplotypes from individuals that were homozygous at all three MHC genes. Next, I identified individuals homozygous at two genes, from these I confirmed the homozygous haplotypes and identified new allele combinations. Lastly, I compared individuals still not assigned with confirmed haplotypes and identified several new allele combinations from remaining individuals. I use allele to refer to unique variants of TLR and MHC genes, and haplotype to refer to a specific combination of alleles at one gene (i.e. the set of alleles a

heterozygote has at TLR genes) or the combination of alleles several genes (i.e. the combination of linked MHC DRB, DQB, and DQA alleles).

Genetic diversity and selection measures. I estimated TLR and MHC genetic diversity by calculating several standard measures of diversity including the number of alleles per gene, haplotype diversity (*H*), number of polymorphic sites (S), nucleotide diversity (π), synonymous nucleotide diversity (π_s), nonsynonymous nucleotide diversity (π_a), and the Watterson estimator of population mutation rate (θ_w) in DnaSP v5. I also measured observed (H_O), expected (H_E) heterozygosity, and deviations from Hardy-Weinberg expectations in Arlequin 3.5 (Excoffier and Lischer 2010). For inbred red wolves, I corrected expected H_E with the average pedigree inbreeding coefficient (*f*) (mean f=0.154; Brzeski et al. 2014) following methods in Hedrick et al. (2002).

To test for selection at TLR and MHC genes, I first used a codon based approach, where I calculated rates of nonsynonymous (d_N) and synonymous (d_S) substitutions according to Nei and Gojobori (1986) with Jukes and Cantor's (1969) correction for multiple hits; I tested d_N-d_S rates for significant differences with a *Z*-test in Mega v.6.0 (Kumar et al. 2001). A d_N-d_S value > 1 indicates positive selection, given an excess of nonsynonymous changes and, while d_N-d_S < 1 indicates purifying selection (Nielsen 2005). I also evaluated how selection varied across TLR and MHC exons because sites involved in pathogen recognition, such as leucine rich regions in TLR genes and antigen-binding sites (ABS) in MHC genes, are expected to be under greater selection pressure than other coding regions. For MHC, I compared d_N-d_S in ABS and non-ABS sites. I also calculated K_A/Ks ratios (the ratio of nonsynonymous substitutions per nonsynonymous site (K_A)) to synonymous substitutions per synonymous site (Ks)) between red wolves and coyotes with a sliding window analysis. I calculated K_A/Ks across all TLR and MHC

genes with a window of 30 base pairs and step size of 10 base pairs in DnaSP v5; I assessed selection patterns with graphical output. The second approach I used to evaluate selection was based on neutral expectations of allele frequencies. To evaluate if TLR and MHC haplotype frequencies were consistent with neutral expectations, I calculated Tajima's D (Tajima 1989) and Fu & Li's D^* and F^* statistics (Fu and Li 1993) in DnaSP v5.

Measurements of immunity and parasite loads. I measured immunity and parasite infections in several ways to ensure I evaluated different components of immune response. First, I measured immunity with bactericidal killing assays (BKA), which estimate an individual's innate immune response by assessing the capacity of blood or serum to kill microbes *ex vivo* (French and Neuman-Lee 2012). I followed methods similar to Tieleman et al. (2005), French et al. (2009) and Garcia et al. (2010), and used two microbes that are destroyed by different immune components: *Escherichia coli* (ATCC #8739, Microbiologics, St Cloud, MN, USA), a gram-negative bacteria killed mostly by complement proteins, and *Candida albicans* (ATCC #10231, Microbiologics, St Cloud, MN, USA), a diploid fungus killed mostly by phagocytosis. I reconstituted lyophilized microbe pellets (1 pellet=10^7 colony forming units (CFU)) in 40 ml of sterile phosphate buffered saline, and prepared a working solution by diluting 2 ml of stock into 8 ml sterile PBS. I mixed serum samples with CO_2-independent media (#18045 Gibco, Carlsbad, CA) enriched with 4 mM L-glutamine (Sigma-Aldrich, St. Louis, MO); the dilution of serum:media was 1:10 for *E. coli* (20µl serum:180µl media) and 1:20 for *C. albicans* (10 µl blood:190 µl media). I added 20 µl of the working bacteria solution to each sample and incubated for 30 minutes at 37°C, after which I spread 50µl in duplicate on tryptic soy agar plates. I incubated plates upside-down at 37°C for 24 and 48 hours, for *E. coli* and *C. albicans* respectively, then counted the CFU per sample. One negative control (media only, no microbes)

and four positive controls (media plus microbes) were included and spread on agar plates in duplicate. I quantified microbial killing capacity as the percent killing per sample relative to control plates, or the mean number of CFU per sample divided by the mean number of CFU on positive control plates (where no killing occurred); I interpreted less microbial killing (i.e. more CFUs on experimental plate) as a weaker immune response. I removed samples which produced a negative or near zero killing capacity because it likely indicated the assay failed; I ran models with and without these samples removed to test model sensitivity. Qualitative results remained the same so I proceeded in leaving suspect samples out of analyses.

I additionally measured immunity by evaluating white blood cell (WBC) and red blood cell (RBC) counts. Whole blood was collected during red wolf processing and blood smears made immediately after collection; whole blood and blood smears were sent overnight to the University of Tennessee's Veterinary Medical Center diagnostic laboratory (Knoxville, TN) for hematology analysis. WBC and RBC counts are relatively easy to collect, provide a gross measure of innate immunity, and can indicate immune capacity by comparing WBC to RBC (Demas et al. 2011, Pedersen and Babayan 2011), where more WBCs indicate greater innate immunity. Their usefulness to evaluate immunocompetence is limited because they represent an individual's hematology at only one point in time, making it difficult to compare individuals exposed to different environmental stressors (Demas et al. 2011), but in conjunction with other measures, blood cell counts can add to an overall picture of immunocompetence (Demas et al. 2011).

Finally, I evaluated pathogen prevalence with several different tests. I collected endoparasites from feces as a measure of pathogen load; fresh feces were collected from wolves and coyotes during processing and sent to University of Tennessee's Veterinary Medical Center

diagnostic laboratory for fecal floats and parasite identification. Infection levels were based on the number of eggs, cysts, or oocysts detected on fecal slides surveyed at 10X magnification across 12 transects, where none = no eggs, cysts, or oocysts detected; low =1-12 eggs, cysts, or oocysts; intermediate ≥12, but eggs, cysts, or oocysts not present on every transect; heavy = eggs, cysts, or oocysts on every transect. I also tested for heartworm (*Dirofilaria immitis*) infections with SNAP® 4Dx Tests (IDEXX Laboratories). Lastly, I tested for canine parvovirus (CPV) and canine distemper virus (CDV) with serological assays, where I sent sera collected from coyotes and wolves to New York State Animal Health Diagnostic Center (Ithaca, NY, USA). I used a hemagglutination inhibition test to detect CPV antibodies (positive titer: ≥20) (Carmichael et al. 1980) and serum neutralization tests (Appel and Robson 1973) to detect CDV (positive titer: >12) antibodies (Clifford et al. 2006). I only tested canids that were not vaccinated prior to sampling because vaccine titers could cause a false positive.

Statistical analyses. I built haplotype networks in TCS 1.21 with amino acid sequences to identify clusters of TLR and MHC alleles that were functionally similar (Clement et al. 2000); this was done to increase statistical power in analyses and to assess functional clusters in addition to nucleotide differences in disease associations. To determine if red wolf and coyote disease susceptibility was influenced by TLR and MHC variation, I used generalized linear mixed effect models (GLMMs) using the R-package lme4 (Bates and Maechler 2010); model error distributions varied depending on the response variable. I ran separate models with each immunological measure as a response variable; BKA percent killing (Gaussian), ratio of WBC:RBC (Gaussian), endoparasite load (Poisson), heartworm infection (positive/negative, binomial), Ehrlichia exposure (positive/negative, logistic), and CPV and CDV exposure (positive/negative, binomial). Fixed variables for GLMMs included four variables previously

shown to influence disease prevalence (Brzeski et al. 2015): age class (pup: less than 12 months old, juvenile: greater than 12 months but under 2 years: adult: greater than 2 years), sex, species, and year collected. Immunogenetic explanatory variables included TLR and MHC heterozygosity (Y/N), haplotypes (assigned value for combination of alleles at each TLR gene or combination of linked alleles at all three MHC genes), clustered allele groups (synonymous or clustered alleles based on TCS analyses), and number of nucleotide SNPs present. I included random effects to control for non-independence between individuals from the same family group (red wolves) or trapping region (coyotes). All models were ranked with AICc and AICc weight *(wi*—Burnham and Anderson 2002*)* and validated by examining residuals and fitted values as suggested by Zuur et al. (2009). I averaged models within Δ2 AICc of the top model using the natural average method (Burnham and Anderson 2002) in R package MuMIn (Bartoń 2009).

I also used contingency tables and odds ratio tests to assess the relationship between heartworm, Ehrlichia, and CPV susceptibility and TLR/MHC haplotypes and clustered allele groups by using presence/absence of parasites and presence or absence of haplotypes or alleles, and calculating the number of red wolves or coyotes in each group. I combined alleles or haplotypes that were present in less than two individuals to represent a group of rare variants.

RESULTS

Genetic diversity and selection measures. I detected multiple TLR and MHC alleles at each TLR and MHC gene sequenced (Table 5.1, 5.2). In TLR genes, there were several alleles at each gene, except for TLR1, which had one synonymous SNP at base 223 (G/A) found only in red wolves (Table 5.3). However, I noted that although I genotyped 22 coyotes at the internal SNP, only one coyote was sequenced across the entire TLR1 exon so it is possible that other variable sites exist in coyotes. There were several nonsynonymous SNPs in each TLR4 exon.

Table 5.1. Toll-like receptor (TLR) genes sequenced in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Assigned allele names, nonsynonymous allele clusters, and frequencies are reported. Underlined samples indicate alleles found in only one species.

Gene (exon)	Allele name	Functional group	Freq. all	Freq. red wolves	Freq. coyote
TLR1	1a	TLR1funct	0.963	0.938	1.000
TLR1	1b	TLR1funct	0.037	0.063	0.000
TLR4(ex1)	4ex1a		0.536	0.444	0.632
TLR4(ex1)	4ex1b		0.436	0.556	0.309
TLR4(ex1)	4ex1c		0.029	0.000	0.059
TLR4(ex2)	4ex2a		0.940	1.000	0.871
TLR4(ex2)	4ex2b		0.052	0.000	0.113
TLR4(ex2)	4ex2c		0.007	0.000	0.016
TLR4(ex3int)	4ex3a		0.742	1.000	0.500
TLR4(ex3int)	4ex3b		0.250	0.047	0.485
TLR4(ex3int)	4ex3c		0.008	0.000	0.015
TLR5(internal)	5a	TLR5functA	0.515	0.795	0.000
TLR5(internal)	5b	TLR5functA	0.191	0.023	0.500
TLR5(internal)	5c	TLR5functA	0.118	0.114	0.333
TLR5(internal)	5d	TLR5functB	0.029	0.045	0.000
TLR5(internal)	5e	TLR5functB	0.029	0.000	0.083
TLR5(internal)	5f	TLR5functA	0.029	0.000	0.083
TLR5(internal)	5g	TLR5functD	0.015	0.000	0.042
TLR5(internal)	5h	TLR5functA	0.015	0.023	0.000
TLR5(internal)	5i	TLR5functC	0.015	0.000	0.042
TLR5(internal)	5j	TLR5functB	0.015	0.000	0.042
TLR5(internal)	5k	TLR5functA	0.015	0.000	0.042
TLR5(internal)	5l	TLR5functB	0.015	0.000	0.042
TLR5(internal)	5m	TLR5functA	0.015	0.000	0.042
TLR6	6a	TLR6functA	0.281	0.455	0.042
TLR6	6b	TLR6functB	0.254	0.333	0.146
TLR6	6c	TLR6functG	0.114	0.000	0.271
TLR6	6d	TLR6functA	0.070	0.106	0.021
TLR6	6e	TLR6functD	0.070	0.015	0.146
TLR6	6f	TLR6functC	0.035	0.030	0.042
TLR6	6g	TLR6functA	0.035	0.000	0.083
TLR6	6h	TLR6functA	0.026	0.045	0.000
TLR6	6i	TLR6functF	0.026	0.000	0.063
TLR6	6j	TLR6functE	0.018	0.000	0.042
TLR6	6k	TLR6functF	0.018	0.000	0.042
TLR6	6l	TLR6functH	0.009	0.015	0.000
TLR6	6m	TLR6functA	0.009	0.000	0.021
TLR6	6n	TLR6functA	0.009	0.000	0.021
TLR6	6o	TLR6functA	0.009	0.000	0.021
TLR6	6p	TLR6functG	0.009	0.000	0.021
TLR6	6q	TLR6functD	0.009	0.000	0.021

Table 5.2. Major histocompatibility complex (MHC) genes sequenced in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Assigned allele names, functional allele clusters, GenBank of previously reported accession numbers, and frequencies for each group are reported. Underlined samples indicate alleles found in only one species.

Gene	Allele name*	Functional group	GenBank accession	All	Red wolf	Coyote
DRB1	09701	DRB1funct1	AF516925	0.293	0.153	0.441
DRB1	06501	DRB1funct2	AF516917	0.214	0.389	0.029
DRB1	06401		AF516919	0.129	0.222	0.029
DRB1	*DRBnew1*		*in progress*	0.086	0.111	0.059
DRB1	03202		AF516916	0.050	0.083	0.015
DRB1	04201		AF343743	0.050	0.000	0.103
DRB1	06601		AF516921	0.043	0.000	0.088
DRB1	06301	DRB1funct1	AF516918	0.021	0.042	0.000
DRB1	CaFa1		AJ459830	0.014	0.000	0.029
DRB1	CaLaDRB2		EU400580	0.014	0.000	0.029
DRB1	*DRBnew2*	DRB1funct3	*in progress*	0.014	0.000	0.029
DRB1	*DRBnew8*	DRB1funct2	*in progress*	0.014	0.000	0.029
DRB1	*DRBnew3*	DRB1funct3	*in progress*	0.007	0.000	0.015
DRB1	04502		AF516922	0.007	0.000	0.015
DRB1	CaLa10	DRB1funct4	AY126665	0.007	0.000	0.015
DRB1	04901		AJ316218/AY126655/JN558751	0.007	0.000	0.015
DRB1	01301		U44778/EU528636	0.007	0.000	0.015
DRB1	*DRBnew7*	DRB1funct4	*in progress*	0.007	0.000	0.015
DRB1	*DRBnew4*		*in progress*	0.007	0.000	0.015
DRB1	*DRBnew6*	DRB1funct4	*in progress*	0.007	0.000	0.015
DQB1	03401		AJ311106	0.257	0.472	0.029
DQB1	00701	DQB1funct1	Y07949/AF043149/AF016907	0.214	0.111	0.324
DQB1	*050v*	DQB1funct2	*in progress*	0.136	0.222	0.044
DQB1	03501	DQB1funct1	AJ311107	0.079	0.111	0.044
DQB1	04301	DQB1funct3	JQ904845	0.050	0.000	0.103
DQB1	05001	DQB1funct2	JQ904834	0.043	0.042	0.044
DQB1	*DQBnew1*		*in progress*	0.043	0.000	0.088
DQB1	*DQBnew2*	DQB1funct3	*in progress*	0.036	0.000	0.074
DQB1	*DQBnew3*		*in progress*	0.036	0.000	0.074
DQB1	02901	DQB1funct3	AF343731	0.021	0.000	0.044
DQB1	*050x*	DQB1funct3	*in progress*	0.021	0.042	0.000
DQB1	00301		AF043151/M90804	0.014	0.000	0.029
DQB1	*DQBnew4*	DQB1funct3	*in progress*	0.014	0.000	0.029
DQB1	008011	DQB1funct3	AF043492/AF043167	0.007	0.000	0.015
DQB1	*DQBnew5*	DQB1funct3	*in progress*	0.007	0.000	0.015
DQB1	00201	DQB1funct3	M90803/AF043148/AF016908	0.007	0.000	0.015
DQB1	*DQBnew7*		*in progress*	0.007	0.000	0.015
DQB1	*DQBnew8*		*in progress*	0.007	0.000	0.015
DQA1	00901		M74908/U44785	0.279	0.472	0.074
DQA1	005011		M74910/U44787	0.229	0.153	0.309
DQA1	01801		AM182471	0.129	0.222	0.029
DQA1	01201		AF343734	0.086	0.111	0.059

(Table 5.2 continued)

Gene	Allele name*	Functional group	GenBank accession	All	Red wolf	Coyote
DQA1	01001		AJ130870	0.079	0.000	0.162
DQA1	CaLu1		DQ777758	0.050	0.000	0.103
DQA1	CaRu1		DQ777755	0.043	0.042	0.044
DQA1	00101		M74907/U44786	0.036	0.000	0.074
DQA1	02401		JQ904831	0.036	0.000	0.074
DQA1	00601		Y07942/U44790	0.014	0.000	0.029
DQA1	01301		AF343735	0.014	0.000	0.029
DQA1	00201		M74909/U75455	0.007	0.000	0.015

*new alleles identified in this study are italicized, alleles confirmed by clones or homozygous are underlined.

TLR4 exon 1 had two SNPs (base 40 G/A, base 71 G/A), resulting in three distinct alleles; red wolves only had two alleles present. TLR4 exon 2 had four SNPs (base 7 G/C, base 13 A/G, base 14 C/T, base 151 G/A), however, all but the SNP at base 151 was due to one unique coyote sequence, resulting in only three haplotypes. Similarly, TLR4 exon 3 had three SNPs (base 166 A/G, base 191 C/T, base 224 A/C), where all but the SNP at base 166 was due to a single coyote sequence, resulting in only three haplotypes. TLR5 had 14 SNPs, resulting in 13 unique nucleotide haplotypes, of which four were nonsynonymous at the protein level. Similarly, TLR6 had 25 SNPs, leading to 17 haplotypes only eight of which were nonsynonymous. With the exception of TLR1, there was greater TLR haplotype diversity in coyotes than red wolves (T-value=2.00, $P<0.036$). For instance, red wolves were monomorphic at TLR4 exon 2 and 3, whereas coyotes had several nonsynonymous haplotypes. I observed the greatest number of haplotypes at TLR5 (4 nonsynonymous) and TLR6 (8 nonsynonymous), despite only sequencing an internal portion of the TLR5 exon (Table 5.1).

Variation at MHC genes was similar between coyotes and red wolves; coyotes had more alleles and greater haplotype diversity than red wolves (T-value=3.50, $P< 0.012$; Table 5.3). All MHC alleles detected were nonsynonymous.

Table 5.3. Gene diversity statistics for TLR and MHC genes sequenced in red wolves and sympatric coyotes. Estimates included number of samples sequenced (N), number of alleles (N_a), haplotype diversity (*H*), number of variable sites (S), nucleotide diversity (π), synonymous (π_s) and nonsynonymous nucleotide diversity (π_a), Watterson's mutation parameter (θ_W), and observed (H_O) and expected heterozygosity (H_E).

Gene	N	Nhap	*H*	S	π	π_s	π_a	θ_W	H_O	H_E
TLR1 internal SNP										
All	54	2	0.072	1	0.00018	0.00086	0.00000	0.00047	0.0741	0.0720
Red wolf	32	2	0.119	1	0.00030	0.00143	0.00000	0.00052	0.1250	0.1007
Coyote	22	1	0.000	0	0.00000	0.00000	0.00000	NA	monomorphic	
TLR4 full exon 1										
All	70	3	0.526	2	0.00593	0.00000	0.00775	0.00390	0.4857	0.5261
Red wolf	36	2	0.501	1	0.00538	0.00000	0.00704	0.00222	0.5556	0.4237
Coyote	34	3	0.509	2	0.00587	0.00000	0.00767	0.00449	0.4118	0.5088
TLR4 full exon 2										
All	67	3	0.112	4	0.00093	0.00000	0.00122	0.00437	0.1194	0.1139
Red wolf	36	1	0.000	0	0.00000	0.00000	0.00000	NA	monomorphic	
Coyote	31	3	0.226	4	0.00189	0.00000	0.00248	0.00507	0.2581	0.2322
TLR4 exon 3 (variable SNP)										
All	66	3	0.400	3	0.00129	0.00040	0.00155	0.00162	0.0758	0.3779
Red wolf	32	1	0.000	0	0.00000	0.00000	0.00000	NA	monomorphic	
Coyote	34	3	0.536	3	0.00183	0.00077	0.00215	0.00184	0.1471	0.5070
TLR5 (internal)										
All	34	13	0.691	14	0.00239	0.00782	0.00040	0.00323	0.5000	0.6910

(Table 5.3 continued)

Gene		N	Nhap	*H*	S	π	π_s	π_a	θ_W	H_O	H_E
	Red wolf	22	5	0.359	4	0.00096	0.00318	0.00014	0.00109	0.3636	0.3041
	Coyote	12	10	0.746	11	0.00260	0.00742	0.00084	0.00350	0.7500	0.7428
TLR6 full exon											
	All	57	17	0.836	24	0.00173	0.00382	0.00115	0.00193	0.7368	0.8361
	Red wolf	33	7	0.678	19	0.00191	0.00444	0.00121	0.00171	0.6970	0.5735
	Coyote	24	15	0.882	23	0.00121	0.00250	0.00086	0.00221	0.7917	0.8821
DRB											
	All	70	20	0.842	47	0.06224	0.03196	0.07251	0.03651	0.7286	0.8419**
	Red wolf	36	6	0.766	44	0.06088	0.03242	0.07056	0.03582	0.7500	0.6345
	Coyote	34	19	0.788	47	0.05040	0.02707	0.05835	0.04205	0.7059	0.7880***
DQB											
	All	70	18	0.859	40	0.05569	0.03250	0.06317	0.02716	0.8143	0.8601***
	Red wolf	36	6	0.709	37	0.04605	0.02794	0.05190	0.02859	0.6944	0.5875
	Coyote	34	17	0.867	40	0.05731	0.03475	0.06457	0.03128	0.9412	0.8674
DQA											
	All	70	12	0.839	9	0.01229	0.00000	0.01593	0.00663	0.7857	0.8420***
	Red wolf	36	5	0.700	6	0.01072	0.00000	0.01387	0.00503	0.6667	0.5921
	Coyote	34	12	0.856	9	0.01336	0.00000	0.01733	0.00764	0.9118	0.8560

* 0.10 > P > 0.05; ** P < 0.05; *** P<= 0.01

I found seven new coyote DLA-DRB1 alleles, four of which I confirmed with cloning, and nine new DLA-DQBI alleles, of which six were confirmed by cloning. I likely detected several new alleles as a result of sequencing coyotes, which have not been the focus of many MHC studies (see Hedrick et al. 2002). With the exception of TLR1 and TLR4, heterozygosity was relatively high at both TLR and MHC genes (Table 5.3).

Selection at TLR genes varied. There was minimal evidence for selection on TLR1 or TLR4 exon 1 and 3 (Table 5.4). Based Fu & Li's D^* and F^* statistics, TLR4 exon 2 may have been under purifying selection in coyotes given both D^* and F^* were significantly negative (Table 5.4). Similarly, both TLR5 and TLR6 showed some evidence of purifying selection, but significance varied by test (Table 5.4). TLR6 selection tests also varied by species, where there was a stronger signal of purifying selection in coyotes and possibly positive selection in red wolves (Table 5.4). Unlike TLR genes, neutrality tests collectively indicated that MHC genes were under positive selection. Nucleotide d_N-d_S estimates were all positive and significantly different from neutral expectations at DLA-DRB1 and DLA-DQA1 genes, and marginally significant at DLA-DQB1 (Table 5.4). ABS were under stronger selection than non-ABS sites at DLA-DRB1 and DLA-DQA1 genes, but d_N-d_S rates across entire MHC exons were still significantly positive (Table 5.4). Similarly, Tajima's D and Fu & Li's D^* and F^* statistics suggested positive selection at all three MHC genes (Table 5.4). The sliding windows analyses with K_A/K_S ratios statistics generally supported that selection varied across exons due to greater selection pressures at leucine rich regions in TLR genes and ABS in MHC genes (Fig. 5.1, 5.2). These regions are predicted to be under stronger selective pressures given their direct, funcationl relationship with antigen binding and antrigen presentation.

Table 5.4. The number of samples sequenced (N), rates of nonsynonymous (d_N) and synonymous (d_S) substitutions with standard error (SE), d_N-d_S, Tajima's D (D) and Fu & Li's D* and F* test results for TLR and MHC genes sequenced in red wolves and sympatric coyotes. For MHC, I compared d_N-d_S at antigen binding sites (ABS) and non-antigen binding sites (nonABS).

Gene	N	d_N (SE)	d_S (SE)	d_N-d_S	D	D*	F*
TLR1 internal SNP							
All	54	0.0000 (0.0000)	0.0009 (0.0009)	-0.9817	-0.7020	0.4876	0.15026
Red wolf	32	0.0000 (0.0000)	0.0014 (0.0014)	-0.9800	-0.5524	0.5231	0.24146
Coyote	22	0.0000 (0.0000)	0.0000 (0.0000)	0.0000	NA	NA	NA
TLR4 full exon 1							
All	70	0.0078 (0.0069)	0.0000 (0.0000)	1.1262	0.7523	0.6605	0.8093
Red wolf	36	0.0071 (0.0069)	0.0000 (0.0000)	1.0077	1.7553*	0.5144	1.0220
Coyote	34	0.0078 (0.0062)	0.0000 (0.0000)	1.2339	0.5098	0.7178	0.7629
TLR4 full exon 2							
All	68	0.0012 (0.0009)	0.0000 (0.0000)	1.3437	-1.4823	-2.8219**	-2.813**
Red wolf	36	0.0000 (0.0000)	0.0000 (0.0000)	0.0000	NA	NA	NA
Coyote	32	0.0025 (0.0019)	0.0000 (0.0000)	1.3460	-1.3499	-2.4429**	-2.4594**
TLR4 exon 3 (internal SNP)							
All	66	0.0016 (0.0014)	0.0004 (0.0004)	0.8110	-0.3478	0.8040	0.5127
Red wolf	32	0.0022 (0.0019)	0.0000 (0.0000)	0.0000	NA	NA	NA
Coyote	34	0.0000 (0.0000)	0.0008 (0.0008)	0.6711	-0.0107	0.8613	0.6922
TLR5 (internal)							
All	34	0.0004 (0.0003)	0.0079 (0.0035)	-2.1717**	-0.7359	-0.6844	-0.8328
Red wolf	22	0.0001 (0.0001)	0.0032 (0.0018)	-1.6627*	-0.2788	1.0172	0.7275
Coyote	12	0.0008 (0.0006)	0.0075 (0.003)	-2.2212**	-0.8707	-0.4333	-0.6566
TLR6							
All	57	0.0001 (0.0004)	0.0038 (0.0014)	-1.9314*	-0.3017	1.4378*	0.9162
Red wolf	33	0.0012 (0.0004)	0.0045 (0.0017)	-1.9037*	0.3658	1.6870**	1.4468
Coyote	24	0.0009 (0.0004)	0.0025 (0.0009)	-1.6501	-1.4745	-2.8516**	-2.8168**
DRB							
All	70	0.0773 (0.0169)	0.0371 (0.0141)	2.6340***	2.1862**	1.4487*	2.1197**
ABS only		0.1573 (0.0354)	0.0413 (0.024)	3.2230***			

(Table 5.4 continued)

Gene		N	d_N (SE)	d_S (SE)	d_N-d_S	D	D^*	F^*
	nonABS		0.0263 (0.0145)	0.0356 (0.0185)	-0.5822			
	Red wolf	36	0.0749 (0.0166)	0.039 (0.0174)	2.3734**	2.3069**	2.08518**	2.5993**
	ABS only		0.1551 (0.0362)	0.0462 (0.0263)	2.6436***			
	nonABS		0.0237 (0.0132)	0.0361 (0.0182)	-0.6890			
	Coyote	34	0.0622 (0.0136)	0.0304 (0.0116)	2.6111**	0.6649	1.4257*	1.3545
	ABS only		0.1246 (0.0290)	0.0358 (0.02)	3.1413***			
	nonABS		0.0221 (0.0115)	0.0278 (0.0148)	-0.4165			
DQB								
	All	70	0.0670 (0.0150)	0.0337 (0.0126)	1.9072*	2.2774**	1.9649**	2.5185**
	ABS only		0.1390 (0.0326)	0.0825 (0.0368)	1.2558			
	nonABS		0.0212 (0.0134)	0.0083 (0.0088)	0.7991			
	Red wolf	36	0.0549 (0.0142)	0.029 (0.0127)	1.5398	1.4752	2.0166**	2.1646**
	ABS only		0.1102 (0.0281)	0.076 (0.0406)	0.7339			
	nonABS		0.0195 (0.0135)	0.0049 (0.005)	0.9694			
	Coyote	34	0.0687 (0.0164)	0.036 (0.0134)	1.9773*	1.8487*	1.8432**	2.2077**
	ABS only		0.1436 (0.0357)	0.0834 (0.0342)	1.5973			
	nonABS		0.0212 (0.0131)	0.011 (0.0111)	0.5714			
DQA								
	All	70	0.0162 (0.0071)	0.0000 (0.0000)	2.2421**	2.0310*	1.2970	1.8519**
	ABS only		0.0768 (0.0344)	0.0000 (0.0000)	2.1889**			
	nonABS		0.0005 (0.0004)	0.0000 (0.0000)	1.0174			
	Red wolf	36	0.0141 (0.0073)	0.0000 (0.0000)	1.883*	2.6856***	1.1462	1.9385**
	ABS only		0.0682 (0.0365)	0.0000 (0.0000)	1.8434*			
	nonABS		0.0000 (0.0000)	0.0000 (0.0000)	0.0000			
	Coyote	34	0.0176 (0.0072)	0.0000 (0.0000)	2.4243**	1.9873*	1.3391*	1.8388**
	ABS only		0.0828 (0.0345)	0.0000 (0.0000)	2.4095**			
	nonABS		0.0009 (0.0009)	0.0000 (0.0000)	0.9941			

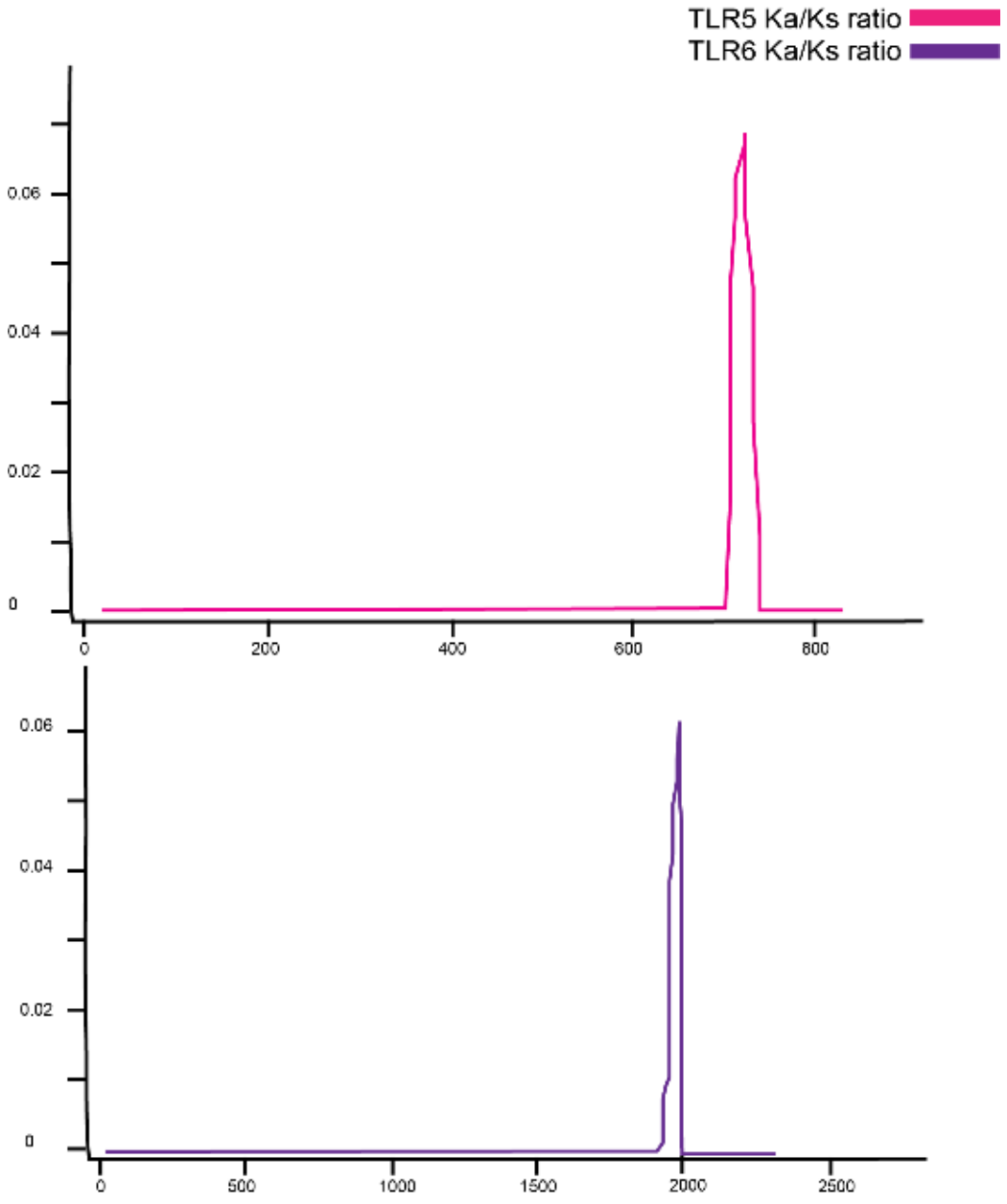

Figure 5.1. *KA/Ks* ratios (the ratio of nonsynonymous substitutions per nonsynonymous site (*KA*)) to synonymous substitutions per synonymous site (*Ks*)) between red wolves (*Canis rufus*) and coyotes (*Canis latrans*) across Toll-like receptor (TLR) genes. *KA/Ks* was calculated across all TLR genes with a sliding window of 30 base pairs and step size of 10 base pairs.

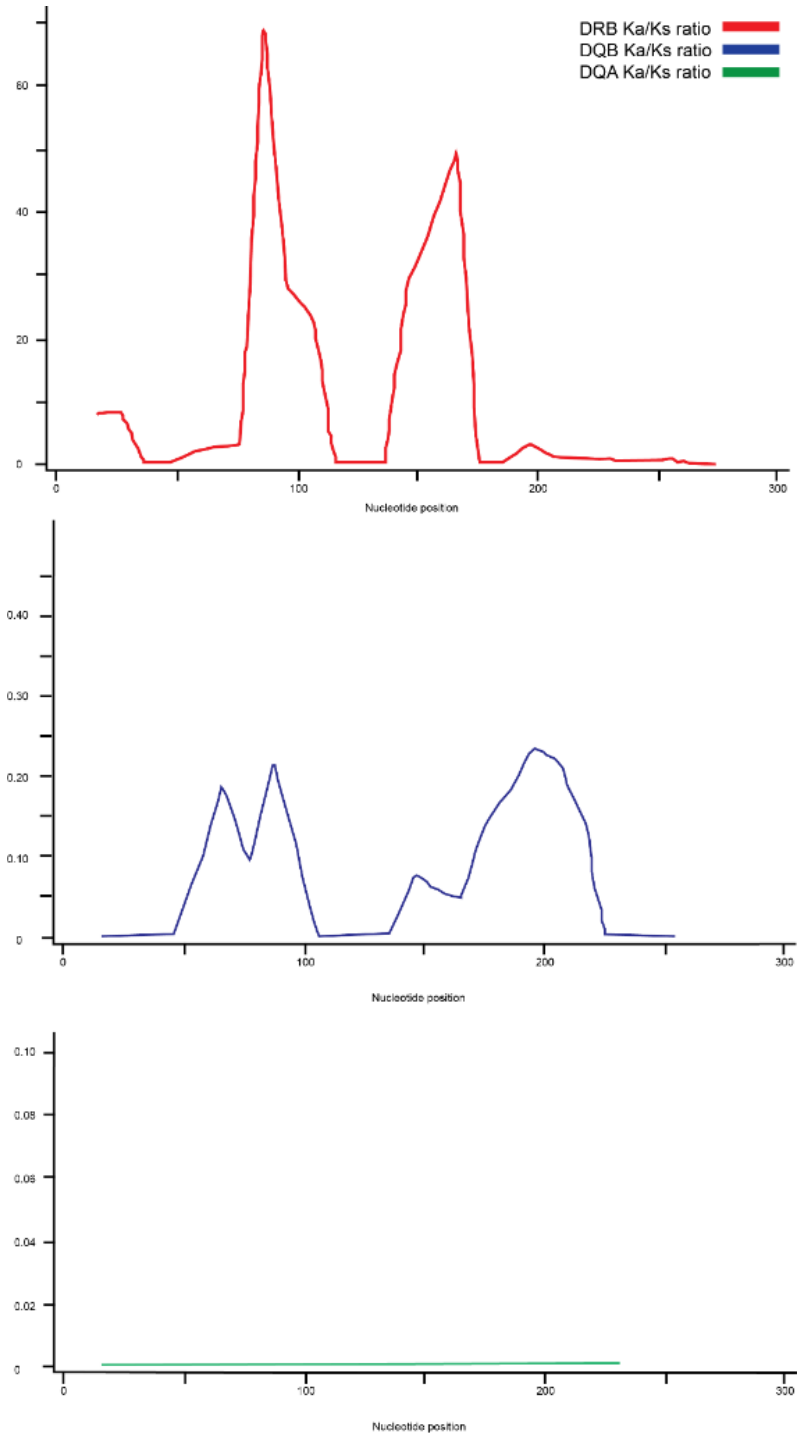

Figure 5.2. *KA/Ks* ratios (the ratio of nonsynonymous substitutions per nonsynonymous site (*KA*)) to synonymous substitutions per synonymous site (*Ks*)) between red wolves (*Canis rufus*) and coyotes (*Canis latrans*) across major histocompatibility complex (MHC) genes. *KA/Ks* was calculated across all MHC genes with a sliding window of 30 base pairs and step size of 10 base pairs.

Measurements of immunity and parasite loads. Average microbial killing capacity was 71.9% and 43.5% for *E. coli* and *C. albicans*, respectively. Species, age class, and sex were not significant predictors of microbial killing capacity but year the assays were performed was important for both *E. coli* (F-statistic=1.12, df=58, P=0.0015) and *C. albicans* (F-statistic=62.34, df=55, P<0.0001). Assay variation, but not average assay killing capacity, differed between 2013 (*E. coli*= 71.7%, *C. albicans*=43.5%) and 2014 (*E. coli*=73.2%, *C. albicans*=46.5%). To control for this in analyses, all BKA models had year included as a random effect. The average ratio of WBC:RBC was 16.4 and was not significantly influenced by species, age classes, sex, or year. Details of pathogen prevalence are presented in Brzeski et al. (2015). Briefly, there were high endoparasite loads in red wolves and coyotes, with 20 different parasite species detected, six of which were non-pathogenic to canids; coyotes had higher endoparasite species diversity (rarefaction projection of red wolves = 16 endoparasite species, coyotes = 31 endoparasite species). Heartworm prevalence was high with a 45% infection rate and all adult red wolves were heartworm positive; given that age class was important in predicting heartworm infection, I included it as a fixed variable in every immunogenetic model. Only 25 canids with immunogenetic data were not vaccinated prior to my sampling period, in which I was able to test CPV and CDV. I found three red wolves and five coyote were positive for CPV exposure based on titer levels; only one red wolf was found to have CDV titers indicative of exposure. I did not include CDV in further analyses given the low exposure rate.

Statistical analyses. TLR1 had only one variable SNP that was nonsynonymous and was therefore dropped from all disease association analyses except for heterozygosity correlations. Based on TCS amino acid haplotype networks, TLR5 nucleotide sequences clustered into four nonsynonymous groups and TLR6 sequences clustered into eight nonsynonymous groups; TLR4

gene sequences did not have any distinct clusters (Fig. D1; Table 5.1). MHC DLA-DQBI and DLA-DRB1 alleles clustered into nine and 15 clusters respectively, where clusters consisted of several nonsynonymous alleles (Fig. D2; Table 5.2); there were no apparent DLA-DQAI clusters.

I found few significant associations between TLR and MHC heterozygosity and immune response or disease prevalence (Table D3). Heterozygous individuals tended to have a lower immune response in BKA assays and lower WBC:RBC ratios than homozygotes (Fig. 5.3). The exceptions to this were TLR4 exon 3, TLR5, and DQAI genes, where heterozygotes had higher immune responses in *C. albican* BKA assays; however, none of these relationships were significant and confidence intervals in GLMM models overlapped zero. The only significant relationship between immune response and heterozygosity was TLR4 exon 1 and *E. coli* BKA assays where heterozygotes had lower killing capacity (Fig. D3). Disease associations with heterozygosity were more varied. Heterozygosity was positively associated with the number of endoparasites at all genes except TLR5 and DRBI; this relationship was significant at TLR4ex3 (Table D3, Fig. D3). Heterozygosity was positively associated with heartworm and Ehrlichia prevalence at all TLR4 exons, but was negatively related at all other genes except DRBI (Table D3, Fig. D3, D4). Again, although heterozygosity was included in these top predictive models, it was not significant and confidence intervals overlapped zero.

I found that several specific alleles were significantly associated with disease (Table D4, D5; Fig. D5, D6). At TLR4 exon 3, individuals with a G at base 166 had higher endoparasite loads than individuals with an A (z-value=2.30, df=85, P=0.022) and also appeared to be more susceptible to CPV (odds ratio 8.48, P=0.005) (Table D5; Fig. D5, D6). Higher endoparasite loads were also associated with several DQB alleles (functional group 3 and DQB new1), but

this relationship was not significant when the top endoparasite-DQB models were averaged. TLR6 functional groupD and TLR6 functional groupE were positively associated with Ehrlichia exposure, whereas TLR6 functional groupF was negatively associated with Ehrlichia (P=0.050; Fig. D6). I did not detect any significant associations between immune measures and specific alleles (Table D4), but having more SNPs at TLR5 was significantly associated with higher BKA *E. coli* killing capacity (Table D5, Fig. D7; estimate=15.76, t-value=3.52, 95% confidence intervals: 7.45-23.87). The number of SNPs an individual had at immune genes was included in a number of other immune and disease top predictive models, but relationships were not significant (Table D6).

DISCUSSION

The ability to cope with newly introduced or evolving parasites is critical for population health and persistence, and can result in parasite-mediated selection shaping immune genes (Areal et al. 2011). Red wolves and coyotes, closely-related and sympatric species with dramatically different demographic histories, may be subject to varying levels of selection when exposed to similar pathogenic pools. To evaluate this, I examined immunogenetic variation and parasite loads in wild red wolves and coyotes and found that both species may have similar evolutionary responses to pathogen pressures. Specifically, innate TLR and adaptive MHC immune genes in wild red wolves and coyotes were polymorphic and displayed evidence of natural selection.

Red wolves, which are endangered and persist in one isolated population, retained polymorphisms at all seven immune genes I sequenced and had several private alleles (Table 5.1, 5.2), despite having much lower immunogenetic variation than coyotes as measured by haplotype diversity. For instance, red wolves had a unique synonymous SNP at TLR1 not present in coyotes. Red wolves also had several unique nucleotide haplotypes at TLR5 and TLR6 genes;

however, red wolves shared all amino acid TLR5 haplotypes with coyotes and had only one unique TLR6 amino acid haplotype. Conversely, coyotes had unique nucleotide and amino acid haplotypes at all TLR genes I sequenced except TLR1. The TLR variation I observed was consistent with other TLR studies on free ranging species in that the number of nucleotide and amino acid alleles I detected was comparable to other populations (Tschirren et al. 2012, Morger et al. 2014, Gavan et al. 2015). Variation at MHC genes was similar to TLR diversity where red wolves had significantly less variation than coyotes, but were still polymorphic and maintained a few private alleles, DRB 06301 and DQB 050x; coyotes had numerous private alleles at all three MHC genes (Table 5.2). Although red wolves shared most haplotypes and alleles with coyotes, the genetic differences between the species underscores that red wolves may have retained unique functional immunogenetic variation despite a severe bottleneck and inbreeding (Brzeski et al. 2014).

The relatively high variability and polymorphism in detected TLR and MHC genes in both species could be the result of parasite-mediated selection (Sommer 2005, Areal et al. 2011). I observed evidence for selection in both gene complexes, but it varied by species and gene; because demography can influence neutrality tests, I only discuss results which several tests validated (Hahn et al. 2002). In general, purifying selection appeared to be acting on TLR4, TLR5, and TLR6 exons because there was an excess of synonymous substitutions and negative selection statistics, although statistics were more significant with coyote sequences and exclusively limited to coyotes at TLR4 (Table 5.4). Purifying selection may indicate that these TLRs are functionally constrained, potentially to preserve biological functions, such as detecting the molecular signature of bacterial flagellin, and may be compromised by nonsynonymous changes (Alcaide and Edwards 2011). Purifying selection has been proposed as the primary

selective force shaping TLR genes due to these constraints in both protein structure and biological function (Yilmaz et al. 2005, Barreiro et al. 2009, Mukherjee et al. 2009, Alcaide and Edwards 2011). Yet recent studies have detected positive TLR selection and suggested that at least non-viral TLRs may not be as functionally constrained as previously thought because of redundancy, therefore nonsynonymous mutations could accumulate without loss of function or compromised immunity (Enard et al. 2010, Areal et al. 2011, Tscherrin 2011, Fornuskova et al. 2014, Gavan et al. 2015). However, my results are concordant with purifying, not positive selection, shaping TLR4, TLR5, and TLR6 diversity in coyotes.

Unlike TLR genes, I found strong evidence of positive selection acting on MHC genes that was statistically significant in both red wolves and coyotes. MHC genes are commonly found to be under balancing selection because of their functional role in antigen recognition and binding (Sommer 2005). Unlike TLRs, which may lose functionality via nonsynonymous mutations, nonsynonymous changes in MHC antigen binding sites may increase the diversity of antigens detected without impeding function, therefore increasing host immunity (Bergström and Gyllensten 1995). Interestingly, red wolves and coyotes had similar selection statistics at all three MHC genes, suggesting that both species may be responding to common pathogen threats on the landscape. Pathogen-mediated balancing selection may outweigh other forces, such as genetic drift, in the red wolf and coyote populations resulting in strong signatures of positive selection at MHC class-II genes.

The strength of selection varied across both TLR and MHC exons, a common phenomenon with functional immune genes given that selection will act differently on portions of the exon directly involved in pathogen recognition (Hedrick 2002, Hughes and Friedman 2008). Both d_N-d_S values and K_A/Ks ratios showed selection was stronger at predicted leucine

rich regions and antigen binding sites, which could be a result of these regions directly influencing pathogen-binding capacity or receptor sensitivity of the gene.

My results demonstrate the critically endangered red wolf has maintained TLR and MHC variation despite the near extinction of the species in the 1970s (Hinton et al. 2013). Immunogenetic variation is crucial for adapting to newly introduced and evolving parasites (Sommer 2005) and my findings underscore the recovery potential of the red wolf, and their potential molecular capacity to combat pathogens on the landscape. Previous research demonstrated that coyotes have more diverse parasite loads than red wolves and may act as a disease source (Brzeski et al. 2015). However, my findings show minimal evidence that pathogens accompanying coyote expansion have limited red wolf recovery. My current results provide a potential mechanism – TLR and MHC variation - in which red wolves are capable of fighting contemporary disease mediated declines.

Wildlife populations that underwent bottlenecks similar to red wolves have been found to be severely depauperate at MHC (Babik et al. 2005, Ploshnitsa et al. 2012, Taylor et al. 2012), yet some populations have retained immune gene diversity through balancing selection (Aguilar et al. 2004, Niskanen et al. 2014, Gavan et al. 2015). Balancing selection can maintain genetic diversity through heterozygous advantage, where heterozygous hosts are able to detect a broader range of parasites (Froeschke and Sommer 2005), or alternatively with negative frequency dependent selection, where rare alleles have a selective advantage (Woolhouse et al. 2002). I found little evidence of heterozygous advantage as heterozygotes tended to have lower bacterial killing capacity and WBC:RBC ratios, and higher disease prevalence (Table D3). This trend was stronger at TLR genes, especially with disease measures, but was also evident in MHC genes and endoparasite associations. In red wolves and coyotes, negative frequency dependent selection

may be the dominant selective pressure on immune genes. For instance, TLR4 exon 3 haplotype was a significant predictor of endoparasites, where individuals with a G instead of A at base 166 had higher endoparasite loads. TLR6 haplotypes were also associated with Ehrlichia exposure, but this relationship was only marginally significant.

Identifying specific haplotypes that may be associated with parasite susceptibility has significant conservation value and is important for red wolf conservation. Notably, red wolves were monomorphic for the G haplotype associated with higher endoparasite load, which have caused high red wolf mortality in the past (Brzeski et al. 2015). However, managing for specific alleles is inappropriate because negative frequency dependent selection leads to haplotypes having a temporary selective advantage, and thus 'good' alleles will vary through time and space. Additionally, parasite exposure often varies as a function of habitat, and managing disease exposure is likely a more direct way to avoid disease mediated population declines than genetic management. However, immune genes are a good indirect measure of the immunological fitness of a population, and the adaptive potential of populations of conservation concern (Sommer 2005) and results presented here demonstrate a small, inbred population can maintain immunogenetic diversity.

My research represents one of the first studies evaluating both innate and adaptive immune genes and disease associations in wild populations. Overall, I documented novel polymorphisms and variable selective pressures that may shape immunogenetic variation in wild populations. While red wolves were less variable than coyotes, they have retained immune gene variation and the potential for adaptive evolution. That I detected similar positive selection at red wolf and coyote MHC genes suggests that two species face similar selective pressures despite variable demography and range sizes.

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CHAPTER 6: INBREEDING AVOIDANCE AND MHC-MEDIATED MATE CHOICE IN ENDANGERED RED WOLVES (*Canis rufus*): WHY RED WOLVES HYBRIDIZE

INTRODUCTION

Mate choice is central to understanding sexual selection in wild populations (Lumley et al. 2015); examining the factors that cause an individual to exclude one available mate and select another elucidates how mate choice can drive phenotypes, fitness, and ultimately population and species persistence. Mate choice contributes to population fitness by removing deleterious genetic mutations, thus improving the genetic health of a population and reducing the risk of extinction (Lumley et al. 2015). Several hypotheses for genetic mate choice have been proposed; the 'good genes' theory suggests mates are selected for genes they carry which increase offspring fitness, such as genes that provide resistance to pathogens (Hamilton and Zuk 1982). Alternatively, mates may be chosen based on genetic compatibility, where offspring genetic diversity is maximized or optimized to local environments (Aeschlimann et al. 2003). These mechanisms are not mutually exclusive given that 'good genes' may be selected that simultaneously increase diversity and heterozygosity (i.e. good genes as heterozygosity advantage'; Brown 1997, Landry et al. 2001). By choosing dissimilar mates and increasing offspring heterozygosity, an individual may also be avoiding kin and inbreeding, which is important for offspring due to the deleterious fitness effects associated with inbreeding (Grob et al 1998, Keller and Waller 2002).

Mate choice and kin recognition is likely facilitated by variation at major histocompatibility complex (MHC) genes (Yamazaki et al. 1976, Grob et al. 1998, Sommer 2005). The MHC is one of the most variable gene complexes known and plays a critical role in cellular immune response. MHC genes encode proteins that detect and present foreign bodies to T-cells, thus an individual's ability to recognize a variety of pathogens is partially dependent on

the number of MHC alleles expressed (Grob et al. 1998). MHC heterozygosity is much higher in natural populations than expected (Hughes and Hughes 1995), possibly due to heterozygotes having the ability to respond to more pathogens (Grob et al. 1998). Indeed, correlations between MHC alleles, haplotypes, or heterozygosity and pathogen resistance have been shown for a number of species (reviewed in Sommer 2005, see Chapter 5). Because MHC variation so strongly affects disease resistance, individuals may select dissimilar mates to produce heterozygous offspring or offspring with advantageous MHC alleles (Landry et al. 2001, Forsberg et al. 2007, Miller et al. 2009, Cutrera et al. 2012). MHC-dependent mate choice has been examined extensively with inbred mice, where mice exhibit preference for MHC dissimilar mates (Yamazaki et al. 1976, 1978, Egid and Brown 1989); wild vertebrate populations have been found to exhibit MHC-dependent mate choice as well (reviewed in Piertney and Oliver 2006).

Mate choice based on MHC variation is likely mediated by individual odor through the expression of molecules with unique binding regions that attach to peptide ligands (Ziegler et al. 2002). Such peptide ligands are excreted in bodily fluids and become free to bind to odorant receptor or vomeronasal gene products and are, therefore, thought to be direct representations of an individual's MHC structure (Ziegler et al. 2002). This creates a mechanism for individuals to avoid mates with similar MHC alleles, such as kin. Kin recognition and inbreeding avoidance can be achieved through MHC mediated mate choice, but there are various other behaviors that facilitate inbreeding avoidance such as familiarity, phenotypic matching, or sex biased dispersal (Pusey and Wolf 1996). Evaluating both MHC and kinship can clarify how these mechanisms impact mate choice in wild populations, and further, how they may influence the persistence of a population.

Endangered wild red wolves (*Canis rufus*) are well suited for evaluating mate choice because they persist in a single small population that is well monitored and has a population wide pedigree (Hinton et al. 2013, Brzeski et al. 2014). Previous analyses show the wild red wolf population is extensively inbred (Brzeski et al. 2014), and they may display inbreeding avoidance behaviors and avoid mating with first-order relatives (Sparkman et al. 2012, Brzeski et al. 2014). For instance, red wolves often disperse from their natal packs and join nonbreeding groups of wolves composed primarily of individuals from different packs or spend time as solitary wolves apart from natal pack memebers (Sparkman et al. 2012). These behaviors break ties with siblings and pack mates, exposing young dispersing wolves to potential mates outside of their natal group, and may explain the relatively few red wolf pairs composed of natal pack members (Sparkman et al. 2012). However, while breaking ties with natal pack members and siblings may reduce procreation with first-order relatives, it may not be effective at reducing overall inbreeding because kinship among non-pack mates is still high within the population of red wolves (Brzeski et al. 2014).

The degree to which canids avoid kin outside of natal packs is uncertain. In general, inbreeding avoidance is more likely to evolve in social and cooperatively breeding species, which encounter relatives more often than non-cooperative species (Pusey and Wolf 1996, Jamieson et al. 2009). Wolves are cooperative breeders, but kin encounter rates for wolves dispersing from natal packs may be low due to large dispersal distances (Geffen et al. 2011). Geffen et al. (2011) compared kin encounter rates and inbreeding avoidance in several canid species and found that individuals from outside natal packs were preferentially selected regardless of relatedness or kin encounter rates. These analyses, similar to Sparkman et al. (2012), recorded kinship as discrete categories of unrelated, half-sibling, or full-sibling/parent-

offspring and did not consider the full range of kinship often observed in wild populations (Geffen et al. 2011). Despite being a small and inbred population, red wolves still exhibit a diversity of kinship relationships (Brzeski et al. 2014) and may exhibit inbreeding avoidance outside of natal packs through mechanisms such as kin recognition or MHC mediated mate-choice.

Selecting mates outside of natal packs may extend to mating with other species when MHC similarity and kinship is very high within a population. Red wolves have hybridized with coyotes (*Canis latrans*), which are closely related (see chapter 2) and very prevalent on the landscape. Inbreeding avoidance could partially explain hybridization if red wolves are avoiding genetically similar mates. Red wolf-coyote hybridization and the resulting introgression of coyote genetic material has been recognized as a biological threat to wild red wolves (Fredrickson and Hedrick 2006). Given that a single and vulnerable population represents the last wild red wolves, assessing how inbreeding avoidance and mate-choice may effect hybridization is an important management objective and has considerable conservation value.

In this study, I evaluated how MHC dissimilarity and kinship influenced red wolf mate choice and hybridization. I tested if observed red wolf pairs departed from the null hypothesis of random mating and if so, whether assortative mating was associated with maximizing or optimizing MHC variation and/or minimizing kinship.

METHODS

Study system. Red wolves are critically endangered canids endemic to the southeastern United States (Phillips and Parker 1988). Although once abundant, persecution and habitat loss confined them to marginal habitat where the threat of extinction *in situ* led United States Fish and Wildlife Service (USFWS) biologists to bring remaining red wolves into captivity (Phillips et al. 2003). A

captive breeding program was established in the 1980s and 14 individuals eventually became the founders of all present day red wolves, however, only 12 are genetically represented in the wild population (Phillips et al. 2003). Starting in 1987, red wolves were reintroduced to Alligator River National Wildlife Refuge in northeastern North Carolina. After reintroductions, the recovery area grew to encompass 1.7 million acres throughout 5 counties (Dare, Tyrrell, Hyde, Beaufort, and Washington), and the red wolf population increased to about 100 individuals (USFWS 2013). However, since 2013, the population has decreased in range and numbers to approximately 50 wild red wolves.

Originally the recovery area had no coyotes, but due to coyote range expansion eastward, coyote-red wolf hybridization was first documented in 1993 (Phillips *et al.* 2003). Hybridization was considered a major threat to red wolf recovery and prompted development of an adaptive management strategy to prevent further introgression of coyote genetic material into the wild red wolf population (Kelly et al. 1999, Stoskopf et al. 2005, Rabon et al. 2013). Under the adaptive management plan, animals considered to be greater than or equal to 87.5% red wolf based on genetic and pedigree assignment (see Miller et al. 2003 for genetic classification details) were allowed to remain in the wild population (Stoskopf et al. 2005). I followed the USFWS criteria and treated all animals determined to be least 87.5% red wolf as part of the wild red wolf population. Part of the adaptive management plan included sterilizing coyote and hybrid mates to act as sterile placeholders with the hope that a red wolf would displace the non-red wolf mate. This resulted in some sterile pairs.

Pairing data. Red wolf management included USFWS Red Wolf Recovery Program biologists trapping red wolves and sympatric coyotes from approximately November to May each year. Wolves and coyotes were captured with soft-catch, off-setting foothold traps and

monitored with radio-collars or surgically implanted abdominal radio transmitters to record reproduction, mortality, and home-range (Phillips *et al.* 2003, USFWS 2013). When wolves were trapped, USFWS biologists took genetic samples and recorded morphological measurements and overall health.

USWFS biologists have closely monitored red wolf pair affiliations due to the threat of hybridization. Red wolf pairs were defined as individuals of breeding age (≥2 years old) who were temporally and spatially associated with one another and had been defending a territory for ≥6 months; pairs were verified through several methods. First, radio-collared wolves and sympatric coyotes were monitored bi-weekly with aerial flights and canids associating in close proximity identified. USFWS biologists confirmed reproduction during the spring (March-May) by locating dens and daybeds of radio-collared paired females to verify the presence of pups from 1991-1999. Starting in 1999, dens were monitored more intensely to implant pups with transponders and take genetic samples to confirm parentage. Pairs observed in the field with offspring were also corroborated though confirming parentage with genetic tests (see Pedigree below). I used the red wolf pedigree in combination with a complete set of chronological and geographical data from the flight records in the Recovery Area to identify and confirm all pairs, both with and without pups, from 1991-2013. Pairs identified and included in analyses were red wolf-red wolf, red wolf-hybrid, and red wolf-coyote.

Intensive management and monitoring has resulted in robust pair data, but mating behavior was also adaptively managed. For instance, if a red wolf was detected with a non-red wolf mate, USFWS biologist would sometimes attempt to trap the wolf and hold it with another trapped wild red wolf to foster red wolf-red wolf pairs. This technique had varying success presumably because red wolves, even if forced together, still made the choice to mate or not. I do

not differentiate between pairs placed together through USFWS management or pairs that formed without interference because all pairs made a choice, at some level to mate, regardless of how they were introduced.

MHC genotyping. I sequenced coding exon 2 in three dog leukocyte antigen (DLA) class II genes: DRB1, DQA1, and DQB1. MHC class II primers have been developed and extensively used in other canid species (Seddon et al. 2002, Kennedy et al. 2007, Wilbe et al. 2009, Kennedy et al. 2011), as well as in captive red wolves, where Hedrick et al. (2000, 2002) examined the class II DRB gene. I used primers as described in Wagner et al. (1996, 1999), and Kennedy et al. (2007; Table E). I extracted DNA from red wolf, hybrid, and coyote blood samples stored in Queen's buffer (Seutin et al. 1991) with QIAGEN® DNeasy blood and tissue kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Polymerase chain reactions (PCR) included 0.25 ng DNA, 0.25 U Taq DNA Polymerase (New England Biolabs),1x Standard buffer (New England Biolabs), 1.5 μM $MgCl_2$, 200 μM dNTPs (QIAGEN), 0.05 μM primer and nanopure water for a final volume of 25 μl. PCR conditions varied by primer pair (Table E1). All PCR product was sent to Beckman Coulter Genomics (Danvers, MA) for bi-directional Sanger sequencing. I cloned and sequenced a subset of samples at MHC genes to confirm the presence of unique or new alleles. For cloning, I followed the same conditions as for sequencing but ran reactions in a 50 μl volume and sent PCR product to MClab (San Francisco, CA) for cloning and sequence verification. All sequences were edited, aligned, and compared with SEQUENCHER v5.1 (Gene Codes Corporation, Ann Arbor, MI USA).

MHC haplotypes were resolved with a stepwise, subtractive approach as described previously (Kennedy et al. 2002a, b). Briefly, I first identified haplotypes from individuals that were homozygous at all three MHC genes which provided known combinations of linked MHC

DRB1, DQB1, and DQA1 alleles. Next, I identified individuals homozygous at two out of the three genes. From these I confirmed the homozygous haplotypes and identified new allele combinations not present in individuals homozygous at all three MHC genes. Lastly, I assessed individuals not assigned complete haplotypes and identified several new allele combinations from remaining individuals. Note that I use the term "allele" to refer to unique gene variants in single MHC genes, and "haplotype" to refer to a specific the combination of linked DRB1, DQB1, and DQA1 alleles.

Pedigree. The red wolf pedigree spans almost 8 generations, where 90% of all red wolf ancestry is known. It was constructed from extensive field data and verified with genetic analyses based on genotyping red wolves at 18 microsatellite loci (Adams 2006); genotypes were used to confirm field determined parentage and assign parentage to individuals with unknown pedigrees (Miller *et al.* 2003, Adams 2006). For individuals with unknown pedigrees, parentage was successfully assigned at the 95% confidence level 95% of the time when one parent was known (~14% of cases) and 88% of the time when neither parent was known (~27% of cases); in most cases (~59%) both parents were identified through field information and verified via genetic methods (see Adams 2006 for details). All known red wolves were included in pedigree construction (Miller *et al.* 2003, Adams 2006). The pedigree is maintained in the program SPARKS (ISIS 2011).

Similarity parameters. I used three different measures to examine if observed mates were more or less similar at MHC genes than expected under random mating. First, I examined the extent of allele sharing to determine if individuals preferentially mated with partners that differed in allele composition, regardless of the functional difference between alleles. If individuals prefer genetically different mates, observed pairs would have lower rates of allele

sharing than expected given random mating. I tested this by calculating the number of alleles (0, 1, or 2) each observed and simulated pair shared (Landry et al. 2001). Next, I examined amino acid differences, which take into account functional differences between MHC alleles. I compared amino acid distance (the total number of nonsynonymous differences) between all pairs of alleles carried by observed and simulated pairs at each gene. I did this across the entire exon as well as just at antigen-binding sites (ABS) of all three MHC genes (Landry et al. 2001, Forsberg et al. 2007, Sin et al. 2015). If individuals prefer mates with functionally different MHC, observed pairs would have greater amino acid distances at MHC genes than expected under random mating. I included amino acid distance only for ABS regions because they are involved in antigen binding and thought to be under stronger selection (Hedrick 2002). For these MHC analyses, I had genotypes for red wolves and all non-red wolf mates (coyotes and hybrids).

To assess inbreeding, I determined if mates were less related than expected based on pedigree kinship. I derived the kinship of observed and potential mates from PMx software (Lacy *et al.* 2011), which is equivalent to the pedigree inbreeding coefficient (*f*) of a pair's hypothetical offspring. Pedigree *f* was defined as the probability that 2 copies of an allele were identical by descent; an individual was inbred if $f > 0$. Red wolf-coyote pairs were assigned a kinship value of zero, given they share no common ancestry. Red wolf-hybrid pairs were removed from the analyses because a hybrid's non-red wolf parents were not maintained in the pedigree, and therefore, they did not have inbreeding coefficients.

Randomization tests. I used randomization tests to statistically evaluate if mean allele sharing, amino acid distance, or kinship of observed pairs was greater or less than expected under random mating (Landry et al. 2001, Forsberg et al. 2007, Miller et al. 2009, Sin et al. 2015). To create a random mating distribution, I dissociated and reassembled all observed mates

for a given time frame (see below) and selected the same number of reassembled pairs at random with replacement. I then calculated the mean allele sharing, amino acid distances, and kinship for the random reassembled pairs and did this 5000 times to create a random distribution. I compared the mean observed pairs to the random distributions; deviations from random mating were considered statistically different than expected if the observed mean fell outside 95% confidence limits. I compared the observed means of all pairs combined, the mean of only red wolf pairs, the mean of red wolf/hybrid/coyote pairs, and the mean of red wolf/coyote pairs only to evaluate how the different groups compared to random mating expectations. I ran separate randomizations for each MHC gene and the sum of shared alleles and amino acid distance for all three genes. All analyses were performed in program R 3.2.2 (R Core Team 2013).

I used randomization tests to evaluate deviations from random mating in several different time periods. First, I evaluated if mate preferences changed over time by running randomizations individually for each year; 1991-2013 for kinship coefficients and 1998-2013 for MHC. I did not have blood samples prior to 1996 for MHC sequencing and there were only 2 and 3 observed pairs with MHC data in 1996 and 1997 respectively (also observed in 1998). Second, I evaluated if mate preference varied during different management phases of the red wolf program, defined as Phase I: 1991-1998, Phase II: 1999-2005, and Phase III: 2006-2013 (Hinton et al. *in press*). During Phase I the USFWS focused on reestablishing wild red wolves, during Phase II the USFWS started their adaptive management plan to control hybridization, and during Phase III population growth stagnated and increased anthropogenic mortality was reported (Hinton et al. *in press*). Each of these phases required different management priorities and had varying red wolf demographics, both of which could influence mating behavior. I ran randomizations for each

phase separately and also combined all observed pairs and ran randomizations with all samples combined into one time frame.

Lastly, I used three different groups to create the random baseline distributions for each time period. For clarity, I refer to each baseline as follows. Baseline 1 consisted of red wolves that bred, and is representative of expected random mating given the actual number of successful breeding pairs; this was considered the most conservative random baseline. Baseline 2 consisted of all paired red wolves in intraspecific pairs (i.e. red wolves associating together that met my pair criteria outlined above), this represented expected random mating within the total potential breeding population of red wolves, without hybridization. Baseline 3 consisted of all paired red wolves, including coyote and hybrid mates, and represented expected random mating of the entire canid breeding population.

RESULTS

I identified a total of 244 breeding pairs where at least one mate was a red wolf; pair number and composition varied by year (Table 6.1). Of the 244 observed pairs, I had MHC and kinship data for 131 and 132 pairs, respectively; sample size varied by year (Table 6.1). MHC variation and polymorphisms are detailed in Chapter 5. Briefly, I observed nine DRB1 alleles, seven DQA1 alleles, and nine DQB1 alleles (Table 6.2). Alleles and haplotypes varied by species but there was substantial overlap (Table 6.3); there were only two private red wolf alleles, one DRB1 and one DQB1 allele. There were two additional DRB1, one DQA1, and two DQB1 alleles present in red wolves and hybrids only. Coyotes had more private alleles with four DRB1, two DQA1, and two DQB1 alleles. There was one DQA1 and one DQB1 allele found only in hybrids. Haplotype variation was similar to allelic diversity, where red wolves had three private haplotypes and coyotes had eight rare haplotypes (Table 6.3). DRB1 alleles differed by

one to 28 amino acids across the exon and one to 25 amino acids at antigen binding sites; DQA1 alleles differed by one to 6 amino acids across the exon and at antigen binding sites; and DQB1 alleles differed by one to eighteen amino acids across the exon and one to fourteen at antigen binding sites.

Table 6.1. Total number of observed red wolf-red wolf (*Canis rufus*) pairs, red wolf-hybrid pairs, red wolf-coyote (*Canis latrans*) pairs, and pairs of red wolves with unknown mates identified in the Red Wolf Recovery Area in northeastern North Caroling from 1991-2013. Total number of observed pairs (N) with data for major histocompatibility complex (MHC; includes red wolves paired with coyotes and hybrids) and for kinship (red wolf pairs only) are reported.

Year	All pairs	Wolf-wolf	Wolf-hybrid	Wolf-coyote	Wolf-Unk.	MHC N	Kinship N
1991	4	4	0	0	0	0	4
1992	3	3	0	0	0	0	3
1993	8	7	0	1	0	0	7
1994	13	11	1	0	1	0	11
1995	11	10	1	0	0	0	10
1996	11	6	4	0	1	2	6
1997	13	9	3	0	1	3	9
1998	14	7	5	2	0	5	7
1999	18	8	8	1	1	10	8
2000	18	7	8	1	2	13	7
2001	26	12	8	1	5	19	12
2002	24	11	11	2	0	23	11
2003	25	20	3	0	2	22	20
2004	23	20	3	0	0	23	20
2005	18	16	1	1	0	20	16
2006	23	15	4	2	2	19	15
2007	26	20	3	3	0	26	20
2008	29	21	0	7	1	19	21
2009	27	17	1	8	1	17	17
2010	26	15	0	11	0	22	15
2011	25	16	1	8	0	7	16
2012	29	19	1	9	0	18	19
2013	13	13	0	0	0	13	13
Total	244	147	39	44	14	131	132

Table 6.2. Total number of major histocompatibility complex (MHC) class II DRB1, DQA1, and DQB1 alleles and frequency of each allele sequenced in red wolves (*Canis rufus*), sympatric coyotes (*Canis latrans*), and red wolf x coyote hybrids. Underlined samples indicate alleles detected only in one species.

Allele*		Total	Red wolf	Hybrid	Coyote
DRB1	03201 (CaRu1)	36	1.00	0.00	0.00
DRB1	06701	67	0.72	0.15	0.13
DRB1	06501 (CaRu2)	43	0.91	0.09	0.00
DRB1	*new1*	22	0.64	0.27	0.09
DRB1	06401 (CaRu4)	6	*0.83*	*0.17*	0.00
DRB1	04901	2	0.00	0.00	1.00
DRB1	11001	2	0.00	0.00	1.00
DRB1	04502 (CaLa15)	1	0.00	0.00	1.00
DRB1	*new8*	1	0.00	0.00	1.00
DQA1	00901	76	0.91	0.07	0.03
DQA1	005011	74	0.73	0.12	0.15
DQA1	012011	20	0.70	0.25	0.05
DQA1	01801	6	0.83	0.17	0.00
DQA1	01001	2	0.00	0.00	1.00
DQA1	CaRu1	1	0.00	1.00	0.00
DQA1	00101	1	0.00	0.00	1.00
DQB1	03401	79	0.95	0.05	0.00
DQB1	00701	65	0.69	0.14	0.17
DQB1	03501	21	0.67	0.24	0.10
DQB1	050v	6	0.83	0.17	0.00
DQB1	050x	3	1.00	0.00	0.00
DQB1	*newCOY2*	3	0.00	0.33	0.67
DQB1	008011	1	0.00	0.00	1.00
DQB1	05001	1	0.00	1.00	0.00
DQB1	*new2*	1	0.00	0.00	1.00

*new alleles identified in this study are italicized.

Table 6.3. Total number of major histocompatibility complex (MHC) class II DRB1, DQA1, and DQB1 haplotypes and frequency of each haplotype sequenced in red wolves (*Canis rufus*), sympatric coyotes (*Canis latrans*), and red wolf x coyote hybrids.

Haplotype	Total	DRB1	DQA1	DQB1	Red wolf	Hybrid	Coyote
C	62	06701	005011	00701	0.73	0.15	0.13
E	43	06501 (CaRu2)	00901	03401	0.91	0.09	0.00
B	30	03201 (CaRu1)	00901	03401	1.00	0.00	0.00
I	20	DRBnew1	012011	03501	0.70	0.25	0.05
A	6	03201 (CaRu1)	005011	03401	1.00	0.00	0.00
G	6	06401 (CaRu4)	01801	050v	0.83	0.17	0.00
D	3	06701	005011	050x	1.00	0.00	0.00
N	2	04901	005011	00701	0.00	0.00	1.00
O	2	11001	00901	newCOY2	0.00	0.00	1.00
CC	1	new8	005011	00701	0.00	0.00	1.00
I1	1	DRBnew1	01001	03501	0.00	0.00	1.00
K	1	06701	01001	new2	0.00	0.00	1.00
L	1	06701	CaRu1	05001	0.00	1.00	0.00
O1	1	DRBnew1	00901	newCOY2	0.00	1.00	0.00
Y1	1	04502 (CaLa15)	00101	008011	0.00	0.00	1.00

For simplicity, I only present MHC randomization results for measures of total MHC similarity during the three management phases and not individually by gene or year; results by gene and year were qualitatively similar to results for each time phase. Also, results were quantitatively similar between random baseline 1 (successful breeding red wolf pairs) and baseline 2 (all intraspecific red wolf pairs; Figure E1-3). Because baseline 1 and baseline 2 were statistically similar and baseline 2 had larger sample sizes, I present randomization results for just baseline 2 and baseline 3 (all paired red wolves, including coyote and hybrid mates).

There was a general trend for observed mates to share fewer alleles then expected (Fig. 6.1), suggesting individuals prefer mates that differ genetically from themselves. This was significant (observed mean outside 95% confidence limits) for red wolf-coyote and red wolf-hybrid pairs regardless of the random baseline distribution or time frame used. Observed red wolf-red wolf pairs also tended to share fewer alleles then expected when I considered random baseline 2. This was true during all time periods, although was not significant. Red wolf-red wolf

pairs did not have fewer shared alleles than expected when considering random baseline 3 (Fig. 6.1). Mean shared alleles of all observed pairs (all pairs: red wolf, hybrid, and coyote) was also lower than expectations (Fig 6.1), this was significant at all time periods, except Phase 1 and Phase 3 when considering the random baseline 3.

Randomization results for MHC amino acid distance were similar across the entire exon and at antigen-binding sites (Fig. 6.2 and Fig. 6.3). Observed red wolf-hybrid and red wolf-coyote pairs were significantly more dissimilar at MHC than expected at all time periods and for both types of random baselines (Fig. 6.2 and Fig. 6.3).

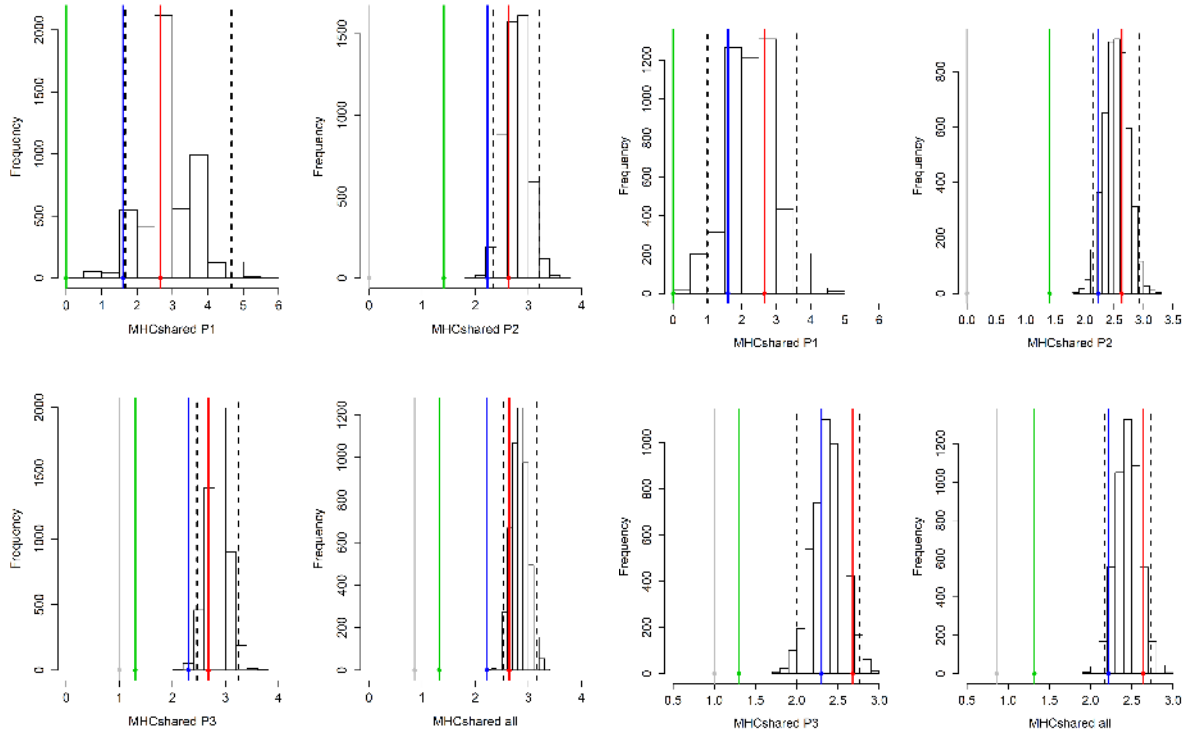

Figure 6.1. Mean number of shared MHC class II genes DRB1, DQB1, and DQA1 alleles of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random baseline 2 (intraspecific red wolf pairs) on the left and baseline 3 (all canid pairs) on the right. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

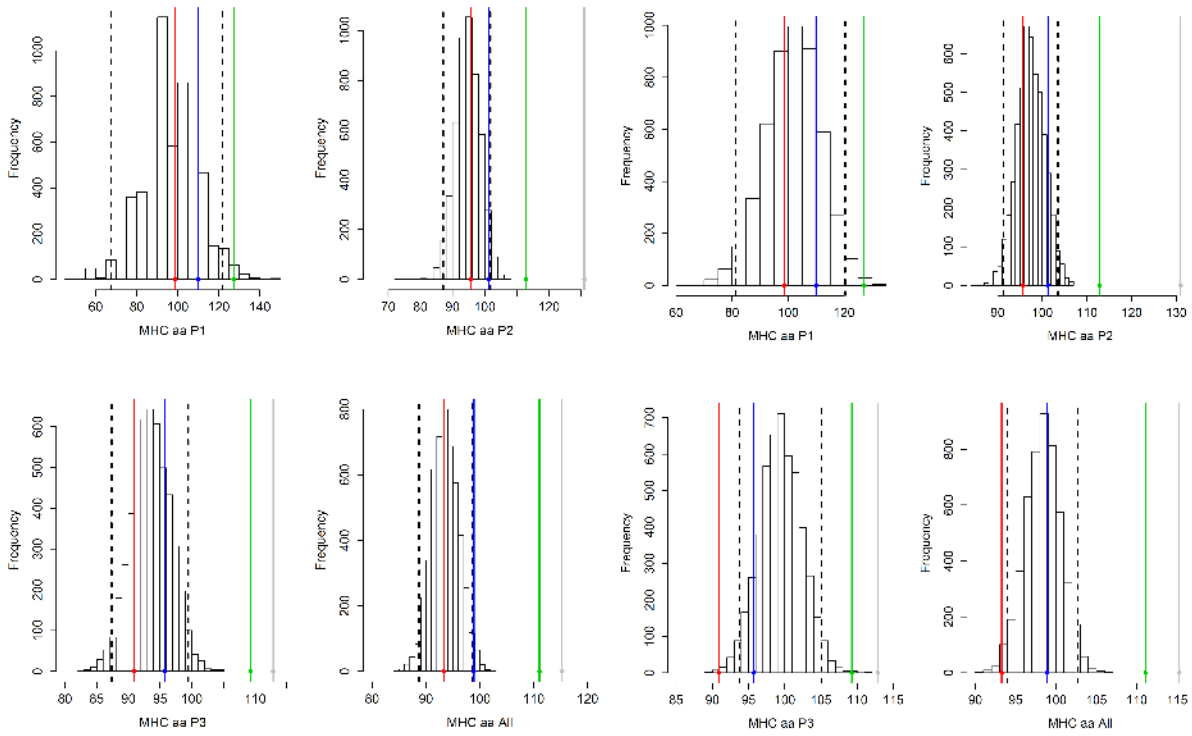

Figure 6.2. Mean combined MHC class II genes DRB1, DQB1, and DQA1 amino acid distance of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random baseline 2 (intraspecific red wolf pairs) on the left and baseline 3 (all canid pairs) on the right. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

Mates also tended to be more dissimilar than expected when considering the average of all observed pairs. However, intraspecific red wolf pairs were not more dissimilar than expected, and when I considered random baseline 3, red wolf pairs were actually significantly more genetically similar than expected (Fig. 6.2 and Fig. 6.3).

During Phase 1, red wolves selected mates with lower than expected kinship, although it did not significantly deviate from random mating expectations (Fig. 6.4). At all other phases, there was little evidence for inbreeding avoidance based on kinship.

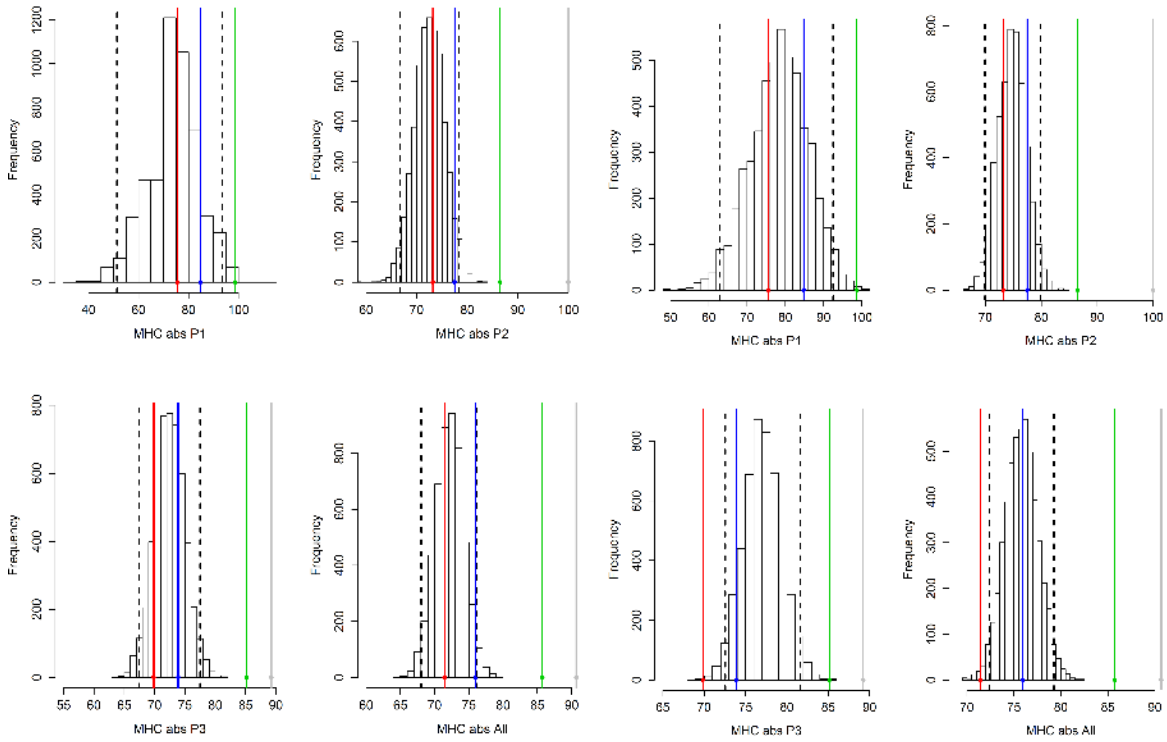

Figure 6.3. Mean MHC class II genes DRB1, DQB1, and DQA1 amino acid distance at antigen-binding sites (abs) of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random baseline 2 (intraspecific red wolf pairs) on the left and baseline 3 (all canids pairs) on the right. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

DISCUSSION

The objective of this study was to determine if endangered red wolves exhibited MHC mediated mate choice or inbreeding avoidance, and how that may influence red wolf-coyote hybridization. I found that overall, there was a trend for red wolves to prefer red wolf and non-red wolf mates with dissimilar MHC genotypes. This pattern was most evident with the number of shared alleles, and although not statistically significant, observed red wolf pairs shared fewer MHC alleles than expected given random mating (Fig. 6.1).

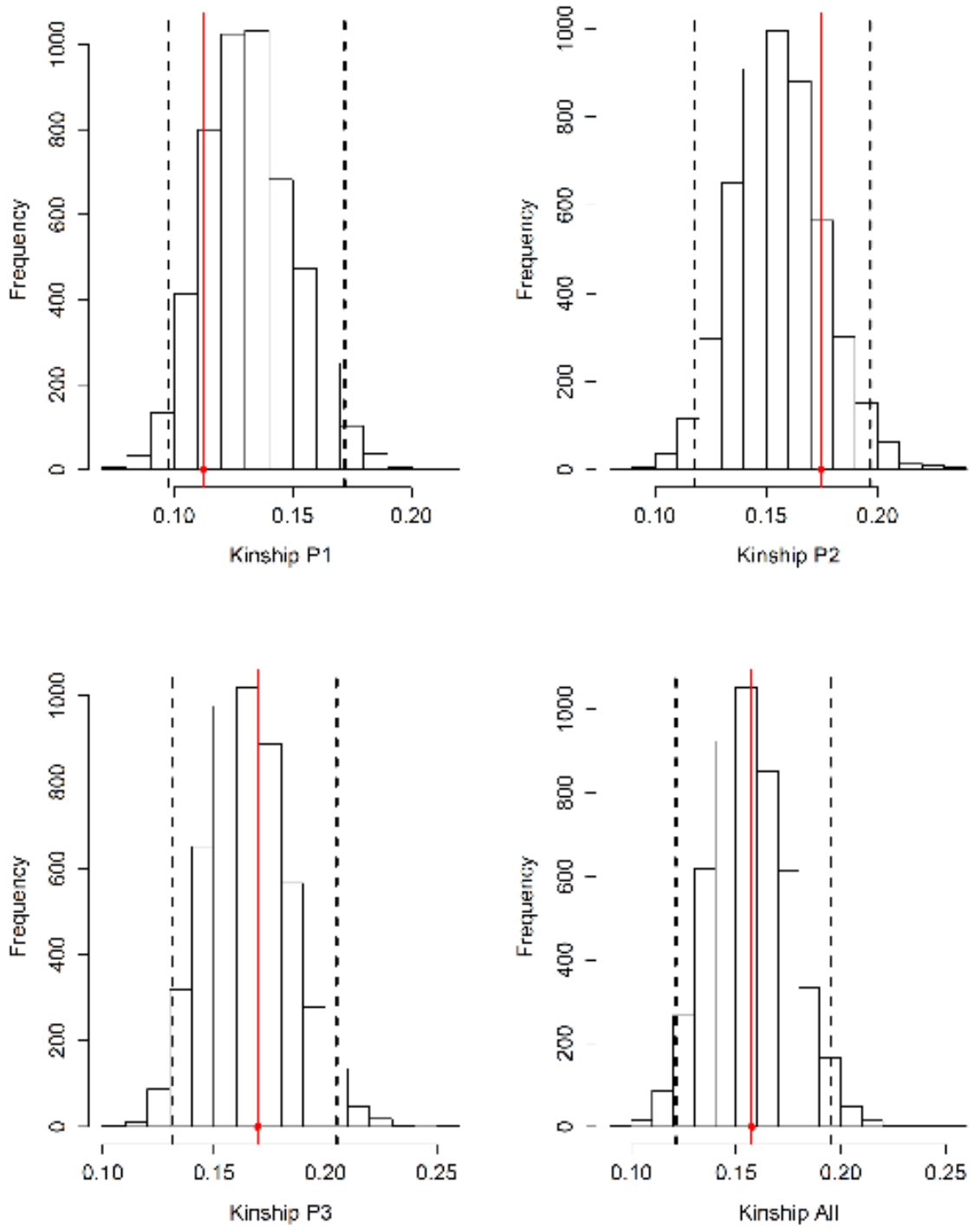

Figure 6.4. Mean pedigree kinship of observed red wolf pairs (red line) compared to random expectations. The frequency distribution (histogram) are mean values generated from 5000 simulations of random pairings of breeding red wolves. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately based on management priorities; Phase 1 (P1: 1991-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

Red wolves may be selecting mates, both within and outside their species, which diversify MHC alleles in progeny and result in offspring with better immune gene repertoires and stronger immune defenses.

Within red wolf pairs, MHC gene function did not appear to influence mate selection, as there was no preference for amino acid distance between intraspecific pairs. Red wolf-red wolf pairs, alternatively, were more similar than expected at amino acid distance when compared to random baseline 3 (random expectations given all canid pairs). I believe this is evidence of red wolf assortative mating, where wolves preferentially mated within species boundaries in regard to functional genetic distances. This is also likely a function of there being less MHC variation in red wolves, which makes it harder to for them to be dissimilar from each other. But when red wolves hybridized, the non-red wolf mate was extremely genetically different; more so than expected given every possible red wolf-coyote or hybrid pairwise comparison. Red wolves may in general prefer to mate with other red wolves but hybridize in situations where extremely dissimilar non-red wolf mates are available, perhaps resulting in offspring with improved fitness due to rare alleles or maximized MHC complexity.

I did not find strong evidence that red wolves avoided inbreeding with kinship metrics (Fig. 6.4), although observed kinship was lower than the expected during Phase I (1991-1998). Phase I was a period of population growth in the wild red wolf population as well as the period with the lowest degree of inbreeding (Brzeski et al. 2014). Kin encounter rates and mean kinship was generally lower during Phase I than in later years as well; during this period red wolf dispersal from natal packs may have been more effective at preventing mating between kin, as demonstrated in other wolf populations (vonHoldt et al. 2008). As pedigree inbreeding and mean

kinship increased through time, natal dispersal may not have been effective in preventing red wolves from mating with close kin.

Interestingly, red wolves currently do not display strong inbreeding depression (Brzeski et al. 2014). This may partially be explained by the detected diversity at MHC genes. Immunogenetic variation is necessary to combat disease and important for overall fitness (Sommer 2005); by maintaining variation at MHC genes, red wolves may be buffered against the deleterious effects of inbreeding and increased homozygosity at other regions of the genome (Charlesworth and Willis 2009). Given red wolves generally preferred mates with fewer shared alleles, they may be maintaining offspring genetic diversity, even with the limited MHC alleles present in the population and high kin encounter rates.

My findings partially support previous studies suggesting that canid inbreeding avoidance is dependent on large dispersal distances and not kin avoidance outside of natal packs (Geffen et al. 2011), because I observed little avoidance of pedigree kinship outside of Phase I. This has negative consequences for endangered wolf populations, and indeed, inbreeding is a major conservation problem for a number of small wolf populations (Liberg et al. 2005, Fredrickson et al. 2007, Räikkönen et al. 2009). But, my data also show that inbreeding may still be avoided by selecting MHC dissimilar mates. Canid inbreeding therefore may be facilitated by MHC-mediated mate choice, even in very inbred populations, and is not solely dependent on avoidance of natal pack members. Hence, to be comprehensive in assessing inbreeding avoidance behavior, functional genetic variation is as important to consider as kinship levels.

While it seems intuitive that mating outside of species boundaries will increase genetic differences, MHC genes actually can be more similar between species then within (Wagner et al. 2012, Lenz et al. 2013). This phenomenon arises due to convergent evolution to similar pathogen

communities or trans-species polymorphisms, where ancient allele lineages are preserved after speciation events (Wegner and Eizaguirre 2012, Lenz et al. 2013). My breeding population of red wolves and coyotes only shared two MHC haplotypes, showing minimal overlap in MHC variation (Table 6.3). Some of the similarity between breeding red wolves and coyote mates was due to coyote introgression because haplotype I, one of the shared haplotypes between coyotes and red wolves, was only present in the wild population and absent from captive red wolves that have not hybridized (Brzeski unpublished data). However, when I compare MHC variation between red wolves and coyotes not present in breeding pairs (Chapter 5, Brzeski unpublished data), there is more genetic overlap between the species. For instance, research that evaluated MHC variation in all red wolves and coyotes trapped during 2013-2014, revealed red wolves only have 2 private alleles and substantial genetic overlap with coyotes (Chapter 5). Thus, convergent evolution, historic hybridization, or ancient allele sharing may explain similarities between all red wolves and coyotes, despite minimal overlap between observed pairs. This is further evidence that hybridization may be facilitated by MHC dissortative mating because there are hybrids and coyotes on the landscape with MHC haplotypes similar to red wolves (Brzeski unpublished data, Chapter 5), but red wolves have only hybridized with exceptionally MHC-dissimilar mates (Fig. 6.1-6.3).

In conclusion, I found evidence for MHC-dissimilar mate preference in the wild red wolf population. Whether this causes hybridization is uncertain, but it does demonstrate that when red wolves hybridize, they are selecting for MHC-dissimilar mates, more so than expected. I only detected inbreeding avoidance per kinship during Phase I of red wolf recovery, which may reflect natal pack member avoidance but not actual kinship avoidance. This highlights the need

to evaluate mate choice over a broad time period to fully understand mating patterns observed in wild populations.

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APPENDIX A: SUPPLEMENTAL FIGURES AND TABLES FROM CHAPTER 2

Table A1. Mitochondrial DNA primer pairs used to amplify a ~450 base pair fragment of the mitochondrial control region in ancient DNA samples. Primers from Leonard et al. (2002).

Pair	Primer ID from Leonard et al. (2002)	Sequence 5'-3'	Product size
pair1F	dogDL-7	TAT TAT ATC CTT ACA TAG GAC	170 bp
pair1R	dogDL-2	GCA AGG GTT GAT GGT TTC TCG	
pair2F	dogDL-1g	GTG CTA TGT CAG TAT CTC CAG G	220 bp
pair2R	dogDL-3	CCC TTA TTG GAC TAA GTG ATA TGC AT	
pair3F	Thr-L	GAA TTC CCC GGT CTT GTA AAC C	250 bp
pair3R	dogDL-5	CAT TAA TGC ACG ACG TAC ATA GG	
pair4F	dogDL-4	GCA TAT CAC TTA GTC CAA TAA GGG	180 bp
pair4R	DL-Hcan	CCT GAG GTA AGA ACC AGA TG	

Table A2. Number of successful PCR reactions for each primer pair (Table A1) used to sequence ancient canid DNA samples, including the number of cloned sequences (in parentheses); one reaction includes both forward and reverse sequences. The total number of base ambiguities observed from amplified product of the same primer pair, overlapping sequences, or cloned samples, with the number of unresolved base ambiguities in parentheses. Each sample underwent two independent DNA extractions, tallies are for the final combined number of sequences, trimmed to 317 basepairs for phylogenetic analyses.

Specimen ID	Primer pair 1 (cloned)	Primer pair 2 (cloned)	Primer pair 3 (cloned)	Primer pair 4 (cloned)	Total ambiguities (unresolved)
CM 038379	7 (0)	7 (0)	8 (8)	8 (7)	7 (5)
UMI 91100	6 (0)	4 (0)	5 (failed)	7 (8)	12 (3)
CM 0006548	8 (0)	4 (0)	8 (5)	8 (8)	7 (4)

Table A3. Sample information including Genbank accession number, putative species, region collected, sample age, and citations, for all for sequences used in gene trees assessing the relationships among canid mitochondrial control region sequences.

Accession number	Putative species	Region	Age	Citation
AF020699	Gray wolf	United States	Contemporary	Pilgram et al. 1998
AF020700	Coyote	United States	Contemporary	Pilgram et al. 1998
AF541876	Coyote/Domestic dog	Southeastern US	Contemporary	Koblmüller et al. 2012
AY163878	Domestic dog	Bolivia	Ancient	Leonard et al. 2002
AY163885	Domestic dog	Peru	Ancient	Leonard et al. 2002
AY163888	Domestic dog	Mexico	Ancient	Leonard et al. 2002
AY812732	Gray wolf, Mexican wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812733	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812734	Gray wolf,	Alaska, Canada	Historic	Leonard et al. 2005
AY812735	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812736	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812737	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812738	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812739	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
AY812740	Gray wolf	Alaska, Canada	Historic	Leonard et al. 2005
EF508156	Coyote	South Carolina	Contemporary	Lance et al. 2007
EF508166	Coyote	South Carolina	Contemporary	Lance et al. 2007
EF508170	Coyote	South Carolina	Contemporary	Lance et al. 2007
EF508172	Coyote	South Carolina	Contemporary	Lance et al. 2007
FM209365	Coyote	Texas	Contemporary	Hailer and Leonard 2008
FM209366	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209367	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209368	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209369	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209370	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209371	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209373	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209374	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209375	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008

(Table A3 continued)

Accession number	Putative species	Region	Age	Citation
FM209376	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209379	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209381	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209382	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209384	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209385	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209386	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209387	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209389	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209390	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209391	Coyote	Nebraska	Contemporary	Hailer and Leonard 2008
FM209392	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209393	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209394	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209395	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209396	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209397	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209398	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209399	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209408	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209411	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209413	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209418	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209420	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
FM209422	Coyote	Texas, Nebraska	Contemporary	Hailer and Leonard 2008
GQ849346	Great Lakes wolf	Midwestern US	Historic	Leonard and Wayne 2008
GQ849360	Great Lakes wolf	Quebec	Contemporary	Leonard and Wayne 2008
GQ849365	Great Lakes wolf	Quebec	Contemporary	Leonard and Wayne 2008
GQ849371	Coyote	Nebraska		Koblmüller et al. 2009
GQ849374	Coyote	Southeastern US	Contemporary	Koblmüller et al. 2012
GQ863718	Coyote	Northeastern US	Contemporary	Kays et al. 2009

(Table A3 continued)

Accession number	Putative species	Region	Age	Citation
GU903017	Red wolf	North Carolina	Contemporary	Bozarth et al. 2011
JN982578	Coyote	Southeastern US	Contemporary	Koblmüller et al. 2012
JN982586	Coyote	Southeastern US	Contemporary	Koblmüller et al. 2012
KM061486	Domestic dog		Contemporary	Duleba et al. 2014
KM061498	Domestic dog		Contemporary	Duleba et al. 2014
KM061528	Domestic dog		Contemporary	Duleba et al. 2014
KM061549	Domestic dog		Contemporary	Duleba et al. 2014
KM061567	Domestic dog		Contemporary	Duleba et al. 2014
KM061583	Domestic dog		Contemporary	Duleba et al. 2014
KM061594	Domestic dog		Contemporary	Duleba et al. 2014

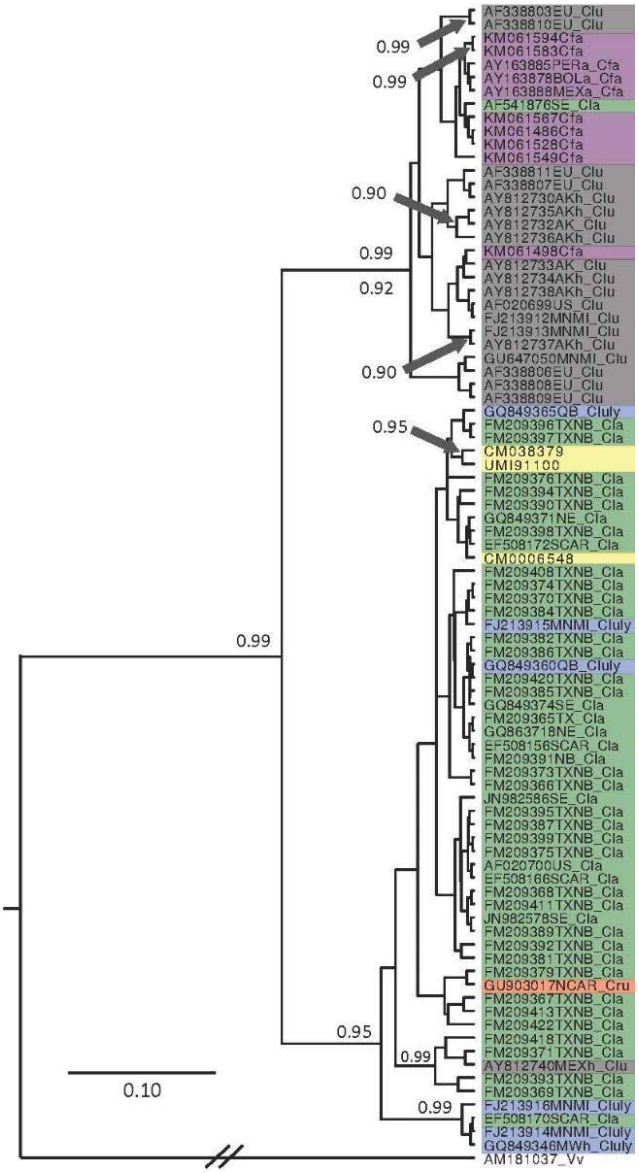

Figure A1. Gene tree showing the relationships among canid mitochondrial control region sequences. The tree is rooted with the fox *Vulpes vulpes*. Bayesian posterior probabilities above 0.90 are listed above the branches or indicated by arrows. Maximum likelihood bootstrap values above 0.90 are listed below the branches. Colors represent the different species with the ancient DNA sequences generated in this study highlighted in yellow. Tip names include the Genbank accession number assigned to each sequence followed by a geographic sampling location, if available, and an abbreviated species name. Historic and ancient DNA sequences downloaded from Genbank are indicated by 'h' and 'a,' respectively. The ancient DNA sequences generated in this study are named according to their museum accession numbers as in Table 1. Other abbreviations are as follows: Clu, *Canis lupus* (gray); Cfa, *Canis familiaris* (purple); Cru, *Canis rufus* (orange); Cla, *Canis latrans* (green); Cluly, *Canis lupus lycaon* (blue); Vv, *Vulpes vulpes* (white); EU, Europe; MEX, Mexico; BOL, Bolivia, PER, Peru; QB, Quebec; US, United States; AK, Alaska; MNMI, Minnesota and Michigan; MW, Midwest USA; NE, New England, USA; SE, Southeast USA; TXNB, Texas and Nebraska, NB, Nebraska; SCAR, South Carolina; NCAR, North Carolina.

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Table B1. Results from a Principle Components Analysis used to create a body size measure for red wolves (*Canis rufus*); the first principle component (PC) encompasses a large portion of overall variance and was used as a response variable in models. Loading values for body size measures and the variance encompassed by each PC are reported.

	PC1	PC2	PC3	PC4	PC5
Body length	0.413	0.468	-0.717	-0.307	0.057
Ear length	0.476	-0.072	-0.148	0.834	-0.226
Tail length	0.352	-0.820	-0.194	-0.359	-0.194
Hind foot length	0.512	-0.037	0.342	-0.022	0.786
Shoulder height	0.465	0.320	0.557	-0.286	-0.538
Proportion of variance	0.620	0.161	0.101	0.078	0.041

Table B2. Pearson correlation coefficients between all numerical explanatory variables, including individual inbreeding coefficients (*f*), parental inbreeding coefficients (dam *f*, sire *f*), parental ages (dam age, sire age), years reproductively available (ry), and territory holders (territory) used in red wolf (*Canis rufus*) fitness models; no variables with a correlation >0.4 were used in the same model, except global models which were included to assess model fit.

	f	dam *f*	sire *f*	dam age	sire age	ry	territory
f	1	-	-	-	-	-	-
dam *f*	0.40	1	-	-	-	-	-
sire *f*	0.40	0.10	1	-	-	-	-
dam age	-0.20	-0.20	0.20	1	-	-	-
sire age	-0.10	-0.01	-0.20	0.40	1		-
ry	-0.24	-0.33	-0.14	0.10	0.10	1	
territory	-0.15	-0.07	-0.08	0.02	-0.03	0.35	1

Table B3. Parameter estimates (β), unconditional standard error (SE), 95% confidence limits (CL), and relative importance (RI) of variables in the final averaged models evaluating the probability of having a territory in wild red wolves (*Canis rufus*).

Explanatory variable	β	SE	CL	RI
Body size (PC1)	2.131	0.849	0.468, 3.795	1.00
Sex	-0.648	0.774	-2.165, 0.869	0.38
Dam age	0.444	0.703	-0.933, 1.823	0.29
Sire age	-0.316	0.671	-1.632, 1.000	0.25
Sex*PC1	-0.054	1.859	-3.698, 3.590	0.07

Table B4. Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating lifetime number of litters (LNL) in endangered red wolves (*Canis rufus*). Years reproductively available abbreviated as ry.

Model	Intercept	Helper	Sex (m)	Damage	*f*	ry	Sire age	Dam *f*	Sire *f*	df	AIC_c	ΔAIC_c	$AIC_c wi$
~helper+dam age+ ry+sire age	-0.36	-0.31		0.46		2.23	-0.35			7	177.5	0.00	0.163
~helper+dam age+ ry+sire age+sire *f*	-0.36	-0.32		0.51		2.16	-0.42		-0.21	8	178.0	0.50	0.126
~ helper+sex+dam age+ry	-0.35	-0.47	-0.23	0.32		2.14				7	178.5	1.03	0.097
~helper+dam age+ry	-0.35	-0.45		0.34		2.17				6	178.5	1.04	0.097
~helper+dam age+ry+sire age+dam *f*	-0.36	-0.35		0.42		2.18	-0.31	-0.10		8	179.2	1.74	0.068
~helper+dam age+*f*+ry+sire age	-0.36	-0.32		0.46	-0.04	2.22	-0.35			8	179.7	2.17	0.055
~helper+sex+dam age+ry+dam *f*	-0.34	-0.50	-0.24	0.29		2.09		-0.16		8	179.7	2.20	0.054
~helper+sex+dam age+*f*+ry+sire age	-0.36	-0.36	-0.21	0.43	-0.02	2.17	-0.32			9	179.8	2.28	0.052
~helper+dam age+ry+dam *f*	-0.35	-0.46		0.31		2.13		-0.15		7	180.0	2.46	0.048
~helper+dam age+ry+sire *f*	-0.35	-0.46		0.35		2.15			-0.09	7	180.5	2.94	0.037
~helper+dam age+*f*+ry	-0.35	-0.48		0.34	-0.10	2.15				7	180.5	2.98	0.037
~helper+sex+dam age+*f*+ry	-0.35	-0.49	-0.23	0.32	-0.06	2.13				8	180.6	3.13	0.034
~helper+sex+dam age+ry+sire *f*	-0.35	-0.47	-0.22	0.33		2.13			-0.05	8	180.7	3.15	0.034

(Table B4 continued)

Model	Intercept	Helper	Sex (m)	Damage	*f*	ry	Sire age	Dam *f*	Sire *f*	df	AIC_c	ΔAIC_c	$AIC_c wi$
~helper+sex+dam age+ry+sire age+dam *f*+sire *f*	-0.35	-0.37	-0.18	0.45		2.11	-0.34	-0.06	-0.14	10	181.0	3.48	0.029
~helper+dam age+f+ry+dam *f*	-0.35	-0.47		0.31	-0.04	2.12		-0.14		8	182.2	4.64	0.016
~helper+ry	-0.36	-0.35				2.23				5	182.8	5.26	0.012
~helper+ry+dam *f*	-0.35	-0.40				2.15		-0.21		6	183.1	5.57	0.010
~helper+sex+ry+sire age+dam *f*	-0.33	-0.43	-0.27			2.12	-0.05	-0.21		8	184.4	6.92	0.005
~helper+sex+ry+sire age	-0.35	-0.38	-0.26			2.21	-0.05			7	184.4	6.93	0.005
~helper+*f*+ry	-0.35	-0.40			-0.12	2.20				6	184.6	7.06	0.005
~helper+ry+sire age	-0.36	-0.34				2.24	-0.04			6	184.9	7.38	0.004
~helper+ry+sire *f*	-0.36	-0.36				2.23			-0.01	6	184.9	7.41	0.004
~helper+ry+sire age+dam *f*	-0.35	-0.38				2.16	-0.04	-0.21		7	185.2	7.70	0.003
~helper+sex+*f*+ry+sire age	-0.35	-0.40	-0.25		-0.07	2.19	-0.04			8	186.5	9.02	0.002
~helper+sex+ry+sire age+sire *f*	-0.35	-0.38	-0.26			2.21	-0.05		0.02	8	186.6	9.13	0.002
~helper+*f*+ry+sire age	-0.35	-0.39			-0.12	2.21	-0.02			7	186.7	9.23	0.002
~helper+ry+sire age+sire *f*	-0.36	-0.34				2.23	-0.04		-0.02	7	187.1	9.55	0.001
null	0.01									3	313.1	135.55	0.000

Random terms: Year of birth and Litter id

Table B5. Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating annual number of litters (ANL) in endangered red wolves (*Canis rufus*).

Model	Intercept	Helper	Sex	Dam	*f*	Territory	Sire	Dam	Sire	df	AIC_c	Δ	AIC_c
~helper+*f*+ty	0.34	-0.15			-	0.38				7	229.6	0.00	0.201
~helper+ty+dam *f*	0.34	-0.14				0.37		-0.09		7	230.0	0.40	0.165
~dam age+ty+sire *f*	0.34			0.10		0.36			-	7	230.9	1.26	0.107
~dam age+ty+dam *f*	0.34			0.08		0.36		-0.04		7	231.4	1.78	0.083
~dam age+f+ty	0.34			0.08	-	0.36				7	231.5	1.82	0.081
~ty+sire age+dam f	0.34					0.36	0.03	-0.06		7	232.5	2.89	0.047
~sex+ty+dam *f*	0.34		-			0.36		-0.05		7	232.6	2.95	0.046
~*f*+ty+sire age	0.34				-	0.37	0.02			7	232.7	3.09	0.043
~ty+sire age+sire *f*	0.34					0.37	0.02		-	7	232.8	3.19	0.041
~sex+*f*+ty	0.34		-		-	0.36				7	233.1	3.47	0.036
~helper+dam	0.34	-0.15		0.12		0.37			-	8	233.4	3.75	0.031
~helper+dam age+*f*+ty	0.34	-0.17		0.09	-	0.37				8	233.8	4.12	0.026
~helper+dam	0.34	-0.15		0.09		0.37		-0.07		8	234.0	4.41	0.022
~helper+ty+sire	0.34	-0.17				0.37	0.07	-0.09		8	234.6	4.98	0.017
~helper+*f*+ty+sire age	0.34	-0.17			-	0.37	0.06			8	234.7	5.04	0.016
~helper+ty+sire age+sire	0.34	-0.14				0.38	0.05		-	8	235.8	6.19	0.009
~sex+dam age+ty+sire *f*	0.34		-	0.10		0.36			-	8	236.7	7.08	0.006
~sex+dam age+*f*+ty	0.34		-	0.08	-	0.36				8	237.2	7.52	0.005
~sex+dam age+ty+dam *f*	0.34		-	0.08		0.36		-0.04		8	237.3	7.67	0.004
~sex+ty+sire age+dam *f*	0.34		-			0.36	0.03	-0.05		8	238.2	8.53	0.003
~sex+*f*+ty+sire age	0.34		-		-	0.36	0.02			8	238.4	8.72	0.003
~sex+ty+sire age+sire *f*	0.34		-			0.37	0.02		-	8	238.4	8.79	0.002
~helper+sex+dam	0.34	-0.15	-	0.12		0.37			-	9	239.0	9.34	0.002
~helper+sex+dam	0.34	-0.17	-	0.09	-	0.37				9	239.2	9.51	0.002
~helper+sex+dam	0.34	-0.16	-	0.09		0.36		-0.07		9	239.5	9.86	0.001
~helper+sex+ty+sire	0.34	-0.18	-			0.36	0.07	-0.09		9	240.1	10.45	0.001
~helper+sex+*f*+ty+sire	0.34	-0.18	-		-	0.37	0.06			9	240.4	10.74	0.001
~helper+sex+ty+sire	0.34	-0.14	-			0.37	0.05		-	9	241.3	11.64	0.001
null	0.35									4	245.9	16.27	0.000
Global model	0.34	-0.19	-	0.10	-	0.35	0.01	-0.06	-	12	254.6	24.98	0.000

Random terms (all

Table B6. Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating the probability of breeding in endangered red wolves (*Canis rufus*). Years reproductively available abbreviated as ry.

Model	Intercept	Helper	Sex	Dam age	*f*	ry	Sire age	Dam*f*	sire *f*	df	AIC_c	Δ AIC_c	AIC_c *wi*
~helper+ry	0.11	-0.69				3.05				5	174.3	0.00	0.176
~helper+dam age+ry	0.11	-0.77		0.36		3.04				6	175.6	1.32	0.091
~helper+ry+dam *f*	0.11	-0.75				3.02		-0.16		6	176.3	2.03	0.064
~helper+*f*+ry	0.11	-0.75			-0.12	3.03				6	176.4	2.06	0.063
~helper+ry+sire *f*	0.11	-0.68				3.05			0.05	6	176.5	2.14	0.060
~helper+ry+sire age	0.11	-0.66				3.05	-0.06			6	176.5	2.14	0.060
~helper+sex+dam age+ry	0.12	-0.83	-0.42	0.36		3.08				7	176.7	2.36	0.054
~helper+sex+ry+dam *f*	0.12	-0.80	-0.42			3.07		-0.14		7	177.4	3.06	0.038
~helper+sex+*f*+ry	0.12	-0.81	-0.42		-0.12	3.08				7	177.4	3.07	0.038
~helper+sex+ry+sire *f*	0.12	-0.73	-0.43			3.09			0.07	7	177.4	3.13	0.037
~helper+sex+ry+sire age	0.12	-0.74	-0.42			3.09	-0.02			7	177.5	3.16	0.036
~helper+dam age+*f*+ry+dam *f*	0.11	-0.81		0.35		3.03		-0.09		7	177.8	3.46	0.031
~helper+dam age+*f*+ry	0.11	-0.81		0.36	-0.08	3.03				7	177.8	3.46	0.031
~helper+*f*+ry+sire *f*	0.11	-0.76			-0.18	3.04			0.13	7	178.5	4.15	0.022
~helper+*f*+ry+dam *f*	0.12	-0.79			-0.09	3.02		-0.13		7	178.5	4.17	0.022
~helper+ry+sire age+dam *f*	0.11	-0.73				3.03	-0.04	-0.15		7	178.5	4.20	0.022

(Table B6 continued)

Model	Intercept	Helper	Sex	Damage	*f*	ry	Sireage	Dam*f*	sire *f*	df	AIC_c	ΔAIC_c	$AIC_c wi$
~helper+*f*+ry+sire age	0.11	-0.73			-0.13	3.04	-0.06			7	178.5	4.22	0.021
~helper+ry+sire age+sire *f*	0.11	-0.66				3.05	-0.05		0.05	7	178.6	4.31	0.020
~helper+sex+dam age+ry+sire age*f*	0.12	-0.74	-0.40	0.44		3.09	-0.21			8	178.7	4.36	0.020
~helper+sex+dam age+*f*+ry	0.12	-0.87	-0.42	0.35	-0.07	3.07				8	178.9	4.54	0.018
~helper+sex+dam age+ry+dam *f*	0.12	-0.86	-0.42	0.34		3.07		-0.07		8	178.9	4.54	0.018
~helper+sex+ry+dam *f*+sire *f*	0.12	-0.79	-0.42			3.07		-0.15	0.08	8	179.5	5.23	0.013
~helper+sex+ry+sire age+dam *f*	0.12	-0.79	-0.42			3.07	-0.01	-0.14		8	179.6	5.27	0.013
~helper+sex+*f*+ry+sire age	0.12	-0.80	-0.42		-0.12	3.08	-0.03			8	179.6	5.28	0.013
~helper+sex+ry+sire age+sire *f*	0.12	-0.73	-0.43			3.09	0.00		0.07	8	179.7	5.34	0.012
~helper+sex+dam age+*f*+ry+sire age	0.13	-0.78	-0.40	0.43	-0.07	3.08	-0.21			9	180.9	6.57	0.007
Global model	0.13	-0.77	-0.40	0.45	-0.04	3.08	-0.23	-0.02	-0.05	11	185.4	11.13	0.001
null	0.13									3	229.5	55.14	0.000

Random terms (all models): Year of birth and Litter id

Table B7. Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating litter size in endangered red wolves (*Canis rufus*).

Model	Intercept	Dam age	Dam *f*	*f*	Sire age	Sire *f*	YOB	df	AIC_c	Δ AIC_c	AIC_c *wi*
~dam age	1.38	-0.20						3	117.5	0.00	0.169
null	1.38							2	118.4	0.90	0.108
~dam age+*f*	1.38	-0.21		-0.14				4	118.5	1.01	0.102
~dam age+sire *f*	1.38	-0.19				-0.03		4	119.6	2.11	0.059
~dam age+sire age	1.38	-0.20			0.01			4	119.7	2.16	0.057
~dam age+dam *f*	1.38	-0.20	-0.01					4	119.7	2.16	0.057
~*f*	1.38			-0.10				3	120.0	2.46	0.049
~YOB	1.38						-0.06	3	120.2	2.72	0.043
~sire *f*	1.38					-0.07		3	120.2	2.72	0.043
~dam *f*	1.38		0.03					3	120.4	2.92	0.039
~sire age	1.38				-0.03			3	120.5	2.95	0.039
~dam age+dam *f*+*f*	1.38	-0.21	0.00	-0.14				5	120.7	3.22	0.034
~dam age+*f*+sire age	1.38	-0.21		-0.14	0.00			5	120.7	3.22	0.034
~dam age+dam *f*+YOB	1.38	-0.19	0.01				-0.06	5	121.6	4.06	0.022
~*f*+YOB	1.38			-0.09			-0.06	4	121.9	4.37	0.019
~dam *f*+*f*	1.38		0.05	-0.10				4	122.0	4.46	0.018
~*f*+sire age	1.38			-0.10	-0.04			4	122.0	4.52	0.018
~*f*+sire *f*	1.38			-0.08		-0.04		4	122.0	4.53	0.018
~sire age+sire *f*	1.38				-0.05	-0.08		4	122.2	4.72	0.016
~dam *f*+sire age	1.38		0.03		-0.02			4	122.6	5.04	0.014
~dam age+dam *f*+*f*+YOB	1.38	-0.21	0.02	-0.13			-0.06	6	122.7	5.21	0.012
~dam *f*+*f*+YOB	1.38		0.06	-0.10			-0.07	5	123.8	6.28	0.007
~*f*+sire *f*+YOB	1.38			-0.08		-0.03	-0.05	5	124.0	6.52	0.006
~*f*+sire age+sire *f*	1.38			-0.08	-0.05	-0.05		5	124.1	6.57	0.006
~sire age+sire *f*+YOB	1.38				-0.05	-0.07	-0.05	5	124.2	6.71	0.006
~*f*+sire age+sire *f*+YOB	1.38			-0.08	-0.05	-0.04	-0.05	6	126.1	8.61	0.002
Global model	1.38	-0.22	0.02	-0.15	0.01	0.04	-0.06	8	127.3	9.78	0.001

Random terms (all models): Pair id

Table B8. Parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating body size in endangered red wolves (*Canis rufus*). Random terms (all models): Year of birth and Pair id.

	Intercept	Sex (m)	Ancestry	Dam age	Dam *f*	*f*	Sire age	Sire *f*	df	AIC_c	Δ AIC_c	AIC_c *wi*
~sex+*f*	0.14	2.26				-0.96			6	423.9	0.00	0.366
~sex+ancestry+*f*	0.12	2.28	-0.40			-0.97			7	425.1	1.20	0.201
~sex+dam age+*f*	0.16	2.25		-0.34		-1.04			7	425.5	1.55	0.169
~sex+*f*+sire age	0.14	2.26				-0.98	-0.09		7	426.8	2.86	0.088
~sex	0.14	2.26							5	428.0	4.08	0.048
~sex+ancestry	0.12	2.27	-0.41						6	429.2	5.28	0.026
~sex+sire *f*	0.17	2.25						-0.29	6	429.9	5.93	0.019
~sex+dam *f*	0.14	2.26			-0.14				6	430.2	6.32	0.016
~sex+dam age	0.15	2.25		-0.21					6	430.4	6.48	0.014
~sex+ancestry+dam *f*	0.12	2.29	-0.54		-0.35				7	430.7	6.79	0.012
~sex+ancestry+sire *f*	0.15	2.27	-0.46					-0.34	7	430.7	6.82	0.012
~sex+sire age	0.14	2.26					0.02		6	430.9	6.94	0.011
~sex+dam age+dam *f*	0.16	2.26		-0.25	-0.22				7	432.4	8.53	0.005
~sex+sire age+sire *f*	0.17	2.25					-0.04	-0.30	7	432.7	8.79	0.005
~sex+ancestry+dam age+dam *f*	0.13	2.29	-0.53	-0.24	-0.42				8	433.0	9.05	0.004
~sex+ancestry+sire age+sire *f*	0.15	2.27	-0.46				0.00	-0.34	8	433.6	9.70	0.003
Global model	0.13	2.27	-0.36	-0.44	-0.01	-1.11	0.21	0.17	11	434.2	10.24	0.002
~null	0.07								4	504.2	80.30	0.000

Table B9. Cox proportional hazard model parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating adult survival in endangered red wolves (*Canis rufus*). Random terms (all models): YOB and Litter id

Model	Dam *f*	Sire *f*	*f*	Dam age	Sire age	Sex (m)	Territory	df	AIC_c	ΔAIC_c	AIC_c *wi*
~sire *f*+territory		-6.78					-2.72	4	853.4	0.00	0.441
~sire *f*+sire age+territory		-7.25			-0.12		-2.69	5	854.0	0.57	0.332
~territory							-2.64	3	858.0	4.51	0.046
~*f*+territory			-2.81				-2.68	4	858.0	4.52	0.046
~*f*+sire age+territory			-2.98		-0.09		-2.66	5	859.2	5.77	0.025
~sex+territory						+	-2.64	4	859.3	5.90	0.023
~sire age+territory					-0.08		-2.63	4	859.4	5.96	0.022
~dam age+territory				0.01			-2.65	4	860.1	6.67	0.016
~dam *f*+territory	0.83						-2.64	4	860.1	6.71	0.015
~*f*+dam age+territory			-2.81	0.01			-2.68	5	860.2	6.73	0.015
Global model	2.33	-7.76	-0.75	0.12	-0.18	+	-2.74	9	860.4	6.91	0.014
~dam *f*+dam age+territory	0.89			0.01			-2.65	5	862.3	8.91	0.005
~sire *f*		-6.18						3	912.9	59.46	0.000
~sire *f*+sex		-6.50				+		4	913.6	60.21	0.000
~sire *f*+sire age		-6.65			-0.12			4	913.7	60.22	0.000
null model								2	916.1	62.62	0.000
~*f*			-1.90					3	917.0	63.58	0.000
~sex						+		3	917.1	63.68	0.000
~sire age					-0.09			3	917.5	64.04	0.000
~*f*+sex			-2.11			+		4	917.9	64.47	0.000
~dam age				-0.04				3	918.0	64.51	0.000
~dam *f*	0.17							3	918.2	64.76	0.000
~*f*+sire age			-1.97		-0.09			4	918.4	64.96	0.000
~*f*+dam age			-1.91	-0.04				4	918.9	65.50	0.000
~dam *f*+sex	-0.31					+		4	919.2	65.81	0.000
~*f*+sire age+sex			-2.19		-0.09	+		5	919.3	65.83	0.000
~*f*+dam age+sex			-2.12	-0.04		+		5	919.9	66.50	0.000
~dam *f*+dam age	0.07			-0.04				4	920.1	66.69	0.000

Table B10. Cox proportional hazard model parameter estimates, corrected delta Akaike information criteria (AIC_c), and AIC_c weights (*wi*) for all models evaluating the probability of juvenile survival (18 months) in endangered red wolves (*Canis rufus*).

Model	Dam *f*	Sire *f*	*f*	Dam age	Sire age	Sex (m)	df	AIC_c	Δ AIC_c	AIC_c *wi*
~sire *f*+sire age		-6.89			-0.01		4	350.0	0.00	0.189
~sex						+	3	350.0	0.02	0.187
~sire *f*		-5.85					3	350.9	0.91	0.120
null model							2	351.1	1.09	0.109
~*f*+sire age			-0.35		0.02		4	352.0	2.06	0.068
~sire *f*+sire age+sex		-6.95			-0.01	+	5	352.5	2.56	0.052
~*f*			-0.35				3	352.9	2.96	0.043
~dam *f*	3.12						3	353.2	3.21	0.038
~sire *f*+sex		-5.85				+	4	353.4	3.44	0.034
~dam age				0.00			3	353.5	3.57	0.032
~sire age					0.00		3	353.7	3.68	0.030
~dam *f*+dam age	3.38			0.00			4	354.6	4.62	0.019
~*f*+sire age+sex			-0.36		0.02	+	5	354.7	4.76	0.017
~*f*+sex			-0.39			+	4	355.4	5.40	0.013
~*f*+dam age			-0.37	0.00			4	355.4	5.40	0.013
~dam *f*+sex	3.07					+	4	355.8	5.86	0.010
~dam age+sex				0.00		+	4	356.1	6.09	0.009
~sire age+sex					0.00	+	4	356.2	6.21	0.008
~dam *f*+dam age+sex	3.37			0.00		+	5	357.3	7.36	0.005
~*f*+dam age+sex			-0.37	0.00		+	5	358.1	8.12	0.003
Global model	5.24	-8.28	1.10	0.00	0.00	+	8	360.6	10.64	0.001

Random terms (all models): YOB and Litter id

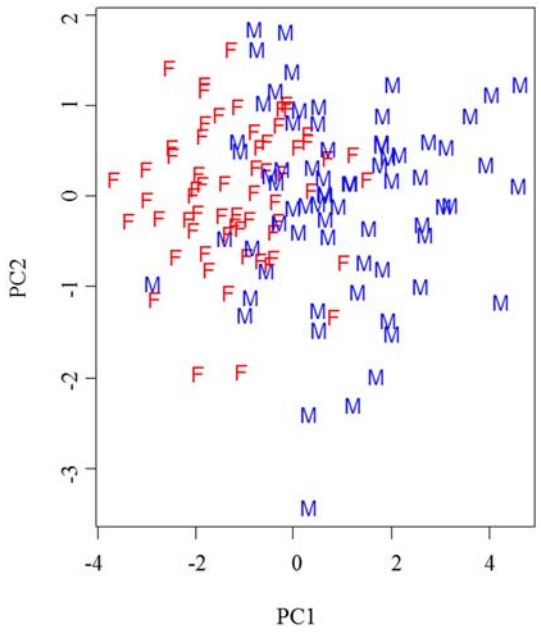

Figure B2. Principle components analysis of body length, hind foot length, shoulder height, ear size and tail length showing how PC1 encompassed overall body size, as demonstrated by the separation of male (blue M) and female (red F) adult red wolves (*Canis rufus*).

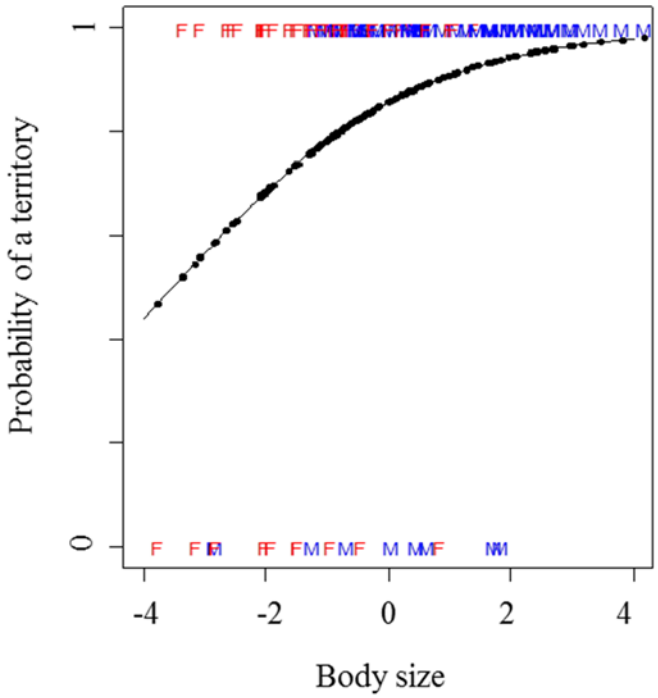

Figure B3. The effect adult red wolf (*Canis rufus*) body size had on the probability of holding a territory for at least one breeding season (1; 0=never held a territory). There was no difference in the probability of holding a territory between female (red F) and males (blue M).

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Table C1. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating endoparasite loads in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~age class	0.0	0.47
~age class + year	1.8	0.20
~age class + sex	2.4	0.14
~age class +species	2.5	0.13
null	6.5	0.02
global	6.6	0.02
~year	8.2	0.01
~species	8.7	0.01
~sex	8.8	0.01
~year + species	10.2	0.00
~year + sex	10.5	0.00
~sex + species	11.1	0.00

Random effect: pack affiliation (red wolves), location (coyote)

Table C2. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating *Spirometra* prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~sex	0.0	0.26
null	0.2	0.24
~year	2.3	0.09
~year + sex	2.3	0.08
~sex + species	2.4	0.08
~species	2.5	0.08
~age class + sex	2.6	0.07
~age class	3.6	0.04
~year + species	4.6	0.03
~age class + year	5.9	0.01
~age class + species	6.1	0.01
global	7.7	0.01

Random term: pack affiliation (red wolf), location (coyote)

Table C3. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating *Sarcocystis* spp .prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~age class	0.0	0.30
null	1.1	0.17
~age class + year	2.0	0.11
~age class + sex	2.5	0.09
~age class + species	2.5	0.09
~species	3.0	0.07
~year	3.1	0.06
~sex	3.3	0.06
~year + species	4.8	0.03
~sex + species	5.4	0.02
~year + sex	5.5	0.02
global	7.2	0.01

Random term: pack affiliation (red wolf), location (coyote)

Table C4. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating *Taeniid* type eggs (*Taenia* spp. and *Echinoccus* spp. eggs are indistinguishable and can only be categorized by egg type)prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
null	0.0	0.22
~year	0.5	0.16
~species	1.0	0.13
~sex	1.5	0.10
~age class	2.4	0.06
~year + species	2.5	0.06
~age class + species	2.5	0.06
~sex + species	2.6	0.06
~year + sex	2.6	0.06
~age class + year	3.0	0.05
~age class + sex	4.2	0.03
global	6.6	0.01

Random term: pack affiliation (red wolf), location (coyote)

Table C5. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating *Uncinaria stenocephala* prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~year	0.0	0.37
~age class + year	1.3	0.20
~year + sex	1.4	0.19
~year + species	1.4	0.19
global	4.6	0.04
null	8.1	0.01
~species	10.0	0.00
~sex	10.3	0.00
~age class	10.4	0.00
~age class + species	12.3	0.00
~sex + species	12.3	0.00
~age class + sex	12.9	0.00

Random term: pack affiliation (red wolf), location (coyote)

Table C6. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating heartworm (*Dirofilaria immitis*) prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~age class + year	0.0	0.50
~age class	2.0	0.19
~age class + species	2.8	0.13
~age class + sex	3.3	0.10
global	3.3	0.09
~year	23.0	0.00
null	23.3	0.00
~species	24.1	0.00
~year + species	24.2	0.00
~sex	25.0	0.00
~year + sex	25.0	0.00
~sex + species	25.9	0.00

Random term: pack affiliation (red wolf), location (coyote)

Table C7. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all generalized linear mixed effect models evaluating *Ehrlichia* spp. prevalence in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~age class	0.0	0.31
~age class + year	1.7	0.13
null	1.7	0.13
~age class + species	1.9	0.12
~age class + sex	2.1	0.11
~year	3.5	0.05
~sex	3.8	0.05
~species	4.0	0.04
~year + sex	5.7	0.02
~year + species	5.8	0.02
global	6.0	0.02
~sex + species	6.1	0.01

Random term: pack affiliation (red wolf), location (coyote)

Table C8. Corrected delta Akaike information criteria (ΔAICc), and AICc weights (*wi*) for all cumulative link mixed models evaluating ectoparasites loads in endangered red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Data was collected in the winters of 2013 and 2014 in northeastern North Carolina. Age classes were defined as pups (under 12 months), juveniles (between 12 and 24 months), and adults (over 24 months).

Model	Δ AICc	AICc *wi*
~year	0.0	0.47
~year + species	1.0	0.29
~year + sex	2.2	0.16
~age class + year	4.0	0.06
global	7.8	0.01
null	11.0	0.00
~species	11.5	0.00
~sex	12.8	0.00
~sex + species	13.2	0.00
~age class	13.9	0.00
~age class + species	15.3	0.00
~age class + sex	15.9	0.00

Random term: pack affiliation (red wolf), location (coyote)

Table C9. Viral pathogens detected in southeastern wildlife populations based on articles containing the words ["United States" AND south* AND (_disease_ OR _parasit*_ OR _pathogen_)] and keyword searches in the following journals: *Journal of Zoo and Wildlife Medicine*, *Journal of Wildlife Disease*, *Journal of Veterinary Medicine, American Journal of Veterinary Research, Journal of Parasitology, American Midland Naturalist*, and *Southeastern Naturalist*.

Pathogen	Detected species	Location[1]	Reference
Bluetongue/epizootic hemorrhagic disease	Black bear	FL	Dunbar et al. 1998
	FL panther	FL	Dunbar et al. 1998
Canine adenovirus -1	Black bear	FL, GA	Pursell et al. 1983, Dunbar et al. 1998
	Gray fox	GA	Gerhold et al. 2007
Canine distemper	Mink	FL	Cunningham et al. 2009
	Gray fox	SE	Davidson et al. 1992, Black et al. 1996, Acton 2008
	Raccoon	FL, NJ, NC	Roscoe 1993, Acton 2008
	Coyote	NC, SC, GA	Holtzman et al. 1992, Acton 2008, Miller et al. 2009
	Red fox (Suspected)	GA	Little et al. 1998
	Domestic dog	FL	Tupler et al. 2012
	Black bear	NC, FL	Dunbar et al. 1998, Acton 2008
Canine enteric coronavirus	Domestic dog	FL	Tupler et al. 2012
Canine hepatitis	Coyote	GA, SC	Holtzman et al. 1992, Miller et al. 2009
Canine influenza (H3N8)	Domestic dog	NC, GA, FL, KY	Anderson et al. 2013
Canine parainfluenza (SC-5)	Coyote	GA	Holtzman et al. 1992
Canine parvovirus	Black bear	FL, NC	Dunbar et al. 1998, Acton 2008
	Coyote	GA, SC (suspected), NC	Holtzman et al. 1992, Acton 2008, Miller et al. 2009
	Domestic dog	FL	Tupler et al. 2012
	Bobcat	NC	Acton 2008
	Gray fox	NC	Acton 2008
	Raccoon	NC	Acton 2008
Equine encephalitis virus	Black bear	FL	Dunbar et al. 1998

(Table C9 continued)

Pathogen	Detected species	Location[1]	Reference
	Coyote	SC	Miller et al. 2009
	Domestic dog	FL	Coffey et al. 2006
Feline calicivirus	FL panther	FL	Roekle et al. 1993
Feline enteric coronavirus/infectious peritonitis	FL panther	FL	Roekle et al. 1993
Feline immunodeficiency virus	FL panther	FL	Roekle et al. 1993
Feline parvovirus	FL panther	FL	Roekle et al. 1993
Pseudorabies virus	Black bear	FL	Pirtle et al. 1986
	Feral pigs	SE	Corn et al. 2004
Rabies	Red and gray fox	SE	Davidson et al. 1992, Kelly and Sleeman 2003, Blanton et al. 2010
	Raccoon	SE	Hubbard 1985, Blanton et al. 2010
	Domestic dog	SE	Blanton et al. 2010
	Domestic cat	SE	Blanton et al. 2010
	Skunk	SE	Blanton et al. 2010
	Bats	SE	Blanton et al. 2010
	Coyote	SE	Krebs et al. 2003, Blanton et al. 2010
	Bobcat	SE	Krebs et al. 2003
	River otter	SE	Krebs et al. 2003
Rotavirus	Domestic dog	FL	Tupler et al. 2012
West Nile Virus	Coyote	SC	Millet et al. 2009
Woodchuck hepatitis	Woodchuck	NC	Cullen et al. 2008

[1]SE indicates a pathogen was detected across the southeastern United States.

Table C10. Endoparasites detected in southeastern wildlife populations based on articles containing the words ["United States" AND south* AND (_disease_ OR _parasit*_ OR _pathogen_)] and keyword searches in the following journals: *Journal of Zoo and Wildlife Medicine*, *Journal of Wildlife Disease*, *Journal of Veterinary Medicine, American Journal of Veterinary Research, Journal of Parasitology, American Midland Naturalist*, and *Southeastern Naturalist*.

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Macracanthoshynchus ingens	Acanthocephala	Black bear	SE	Crum et al. 1978, Foster et al. 2004
		Bobcat	WV, GA	Watson et al. 1981
		Raccoon	SE	Jordan and Hayes 1959, Harkema and Miller 1964, Bafundo et al. 1980
		Mink	FL	Foster et al. 2007
Moniliformis clarki	Acanthocephala	Southern flying squirrels	GA	Pung et al. 2000
Polymorphus brevis	Acanthocephala	Mink	FL	Foster et al. 2007
Otodectes Cynotis	Arthropod	Red fox	GA	Little et al. 1998
Sarcoptes scabiei	Arthropod	Red fox	SE	Little et al. 1998, Kelly and Sleeman 2003
		Coyote	TX	Pence et al. 1983, Pence and Windberg 1994
Atriotaenia procyonis	Cestode	Raccoon	SE	Harkema and Miller 1964, Bafundo et al. 1980
Cittotaenia variabilis	Cestode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Dipylidium caninum	Cestode	Domestic dog	FL	Tupler et al. 2012
Mesocestoides variabilis	Cestode	Bobcat	WV, GA	Watson et al. 1981
		Raccoon	NC, SC, GA, FL, TN	Harkema and Miller 1964, Bafundo et al. 1980
Moniezia	Cestode	White-tailed deer	SE	Prestwood 1971
Raillietina salmoni	Cestode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Spirometra mansonoides	Cestode	Black bear	FL, GA	Crum et al. 1978
		Bobcat	WV, GA	Watson et al. 1981

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
		Raccoon	SE	Harkema and Miller 1964
Taenia spp.	Cestode	Coyote	GA	Holtzman et al. 1992
		Bobcat	WV, GA	Watson et al. 1981
		White-tailed deer	SE	Prestwood 1971
Taenia pisiformis	Cestode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Adelina spp.	Coccidia	Cotton rats	AL	Barnard et al. 1974
Eimeria spp.	Coccidia	Cotton rats	AL	Barnard et al. 1974
Isospora spp.	Coccidia	Coyote	SC	Miller et al. 2009
		Cotton rats	AL	Barnard et al. 1974
Encephalitozoon cuniculi	Fungi	Domestic dog	FL, TX	Snowden et al. 1999, Clay and Rivas 2013
Acanthocheilonema procyoni	Nematode	Raccoon	GA	Pung et al. 1996
Aelurostrongylus spp.	Nematode	Raccoon	NC, SC	Harkema and Miller 1964
Ancylostoma caninum	Nematode	Coyote	GA, SC	Holtzman et al. 1992, Lee et al. 1993, Miller et al. 2009
		Black bear	TN, FL, GA	Crum et al. 1978, Foster et al. 2004
		Domestic dog	SE	Mohamed et al. 2009, Tupler et al. 2012
Ancylostoma braziliense		Bobcat	WV, GA	Watson et al. 1981
		Domestic cat	FL	Liotta et al. 2012
Ancylostoma tubaeforme		Bobcat	WV, GA	Watson et al. 1981
		Black bear	FL	Foster et al. 2004
		Domestic cat	FL	Liotta et al. 2012
Arthrocephalus lotoris	Nematode	Black bear	SE	Crum et al. 1978
		Raccoon	NC, SC, GA, VA, TN	Jordan and Hayes 1959, Harkema and Miller 1964, Bafundo et al. 1980
Ascaris spp.	Nematode	Domestic dog	FL	Tupler et al. 2012

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Baylisascaris spp.	Nematode	Raccoon	SE	Bafundo et al. 1980, Kazacos 2001, Souza McCleery et al. 2005, Eberhard et al. 2003, Souza et al. 2009, Blizzard et al. 2010, Hernandez et al. 2013
Baylisascaris transfuga	Nematode	Black bear	FL	Foster et al. 2004
Baylisascaris procyoni	Nematode	Black bear	VA, WV, NC	Crum et al. 1978, Duncan et al. 1999
Capillaria spp.	Nematode	Raccoon	VA, SC, LA, PN	Pence 1975, Hamir and Rupprechtt 1998
Capillaria aerophila	Nematode	Black bear	NC, TN	Crum et al. 1978, Foster et al. 2004
		Bobcat	WV, GA	Watson et al. 1981
		Mink	FL	Foster et al. 2007
Capillaria mustelorum	Nematode	Raccoon	NC, GA	Harkema and Miller 1964
Capillaria plica	Nematode	Raccoon	NC, SC, GA, VA	Harkema and Miller 1964
Capillaria putorii	Nematode	Black bear	SE	Crum et al. 1978, Foster et al. 2004
		Bobcat	WV, GA	Watson et al. 1981
Citellinema spp.	Nematode	Bobcat	WV, GA	Watson et al. 1981
Citellinema bifurcatum	Nematode	Southern flying squirrels	GA	Pung et al. 2000
Crenosoma spp	Nematode	Black bear	VA, WV	Crum et al. 1978
Crenosoma goblei		Raccoon	NC, SC, GA	Harkema and Miller 1964
Cosmocephalus spp.	Nematode	Raccoon	SC	Harkema and Miller 1964
Cyathospirura spp.	Nematode	Black bear	VA, WV	Crum et al. 1978
		Bobcat	WV, GA	Watson et al. 1981
Cylicospirura felineus	Nematode	Bobcat	WV, GA	Watson et al. 1981
Cyrnea spp.	Nematode	Bobcat	WV, GA	Watson et al. 1981
Dermatoxys veligera	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Dioctophyma renale	Nematode	Raccoon	NC	Harkema and Miller 1964

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Dirofilaria immitis	Nematode	Coyote	SE	Croswell et al. 1977, Custer and Pence 1981, King and Bohing 1984, Holtzman et al. 1992, Lee et al. 2007, Miller et al. 2009
		Red fox	MO, AR	Wixsom et al. 1991
		Gray fox	AR, AL, MS, GA	Simmons et al. 1980, King and Bohing 1984
		Raccoon	VA	Synder et al. 1989
		Black bear	NC	Crum et al. 1978
		Domestic dog	SE	Rothstein et al. 1961, Levy et al. 2007
		Domestic cat	NC	Atkins et al. 2005
Dirofilaria lutrae	Nematode	Mink	FL	Foster et al. 2007
Dirofilaria scapiceps	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Dirofilaria tenuis	Nematode	Raccoon	GA	Pung et al. 1996
Dracunculus insignis	Nematode	Gray fox	GA	Davidson et al. 1992
		Raccoon	NC, SC, FL	Harkema and Miller 1964
Echinococcus spp	Nematode	Domestic dog		Franklin and Ward 1953
Echinococcus granulosus		Domestic dog	Lower MS valley	Ward 1965
Gnathostoma spp	Nematode	Black bear	VA, WV	Crum et al. 1978
Gnathostoma procyonis	Nematode	Raccoon	SE	Jordan and Hayes 1959, Harkema and Miller 1964, Bafundo et al. 1980, Lockhart 2007
Gongylonema pulchrum	Nematode	Black bear	SE	Crum et al. 1978, Kirkpatrick et al. 1986
		Eastern cottontail rabbit	AL, SC	Andrews and Davidson 1980
		Black bear	FL	Foster et al. 2004
		Bobcat	WV, GA	Watson et al. 1981
Lagochilascaris sprenti	Nematode	Opossum	LA	Bowman et al. 1983

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Longistriata noviberiae	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Mansonella llewellyn	Nematode	Raccoon	GA	Pung et al. 1996
Metathelazia spp	Nematode	Bobcat	WV, GA	Watson et al. 1981
Molineus barbatus	Nematode	Black bear	SE	Crum et al. 1978, Foster et al. 2004
		Bobcat	WV, GA	Watson et al. 1981
		Raccoon	NC, SC, GA, VA, TN	Jordan and Hayes 1959, Harkema and Miller 1964, Bafundo et al. 1980
Molineus patens	Nematode	Mink	FL	Foster et al. 2007
Obeliscoides cuniculi	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Oesophagostomum spp	Nematode	Bobcat	WV, GA	Watson et al. 1981
Oslerus rostratus	Nematode	Bobcat	WV, GA	Watson et al. 1981
Oxyuriidae	Nematode	Bobcat	WV, GA	Watson et al. 1981
Passalurus ambiguus	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Physaloptera spp	Nematode	Coyote	GA	Holtzman et al. 1992
		Black bear	SE	Crum et al. 1978
		Mink	FL	Foster et al. 2007
Physaloptera maxillaris	Nematode	Raccoon	GA	Jordan and Hayes 1959
Physaloptera rara	Nematode	Bobcat	WV, GA	Watson et al. 1981
		Raccoon	NC, SC, GA, VA, TN	Harkema and Miller 1964, Bafundo et al. 1980
Rictularia spp.	Nematode	Bobcat	WV, GA	Watson et al. 1981
Syphacia thompsoni	Nematode	Southern flying squirrels	GA	Pung et al. 2000
Spirocerca lupi	Nematode	Domestic dog	SE	Bailey et al. 1963, 1964, Dixon and McCue 1967
Strongyloides spp.	Nematode	Black bear	SE	Crum et al. 1978, Foster et al. 2004
		Mink	FL	Foster et al. 2007

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Strongyloides robustus		Southern flying squirrels	GA	Pung et al. 2000
Trichinella spp.	Nematode	Feral pigs	NC	Sandfoss et al. 2011
		Coyote	TX	Pozio et al. 2001
		Black bear	TN	Schad et al. 1986
Trichostrongylus affinis	Nematode	Bobcat	WV, GA	Watson et al. 1981
		Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Trichostrongylus axei	Nematode	Bobcat	WV, GA	Watson et al. 1981
Trichostrongylus calcaratus	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Trichuris leporis	Nematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Toxocara sp.	Nematode	Red fox	SC	Lee et al. 1993
		Coyote	SC	Lee et al. 1993
		Domestic dog	SE	Mohamed et al. 2009
Toxascaris leonia	Nematode	Bobcat	WV, GA	Watson et al. 1981
		Raccoon	GA	Blizzard et al. 2010
Toxocara mystax		Bobcat	WV, GA	Watson et al. 1981
Trichuris spp.	Nematode	Red fox	SC	Lee et al. 1993
		Coyote	SC	Lee et al. 1993, Miller et al. 2009
		Domestic dog	SE	Tupler et al. 2012
Trichuris odocoileu	Nematode	White-tailed deer	GA	Knight 1983
Troglostrongylus wilsoni	Nematode	Bobcat	WV, GA	Watson et al. 1981
Uncinaria spp.	Nematode	Bobcat	WV, GA	Watson et al. 1981
Vigisospirura potekhina	Nematode	Bobcat	WV, GA	Watson et al. 1981
Babesia spp.	Protozoa	Bobcat	GA	Shock et al. 2013
		Domestic dog	VA, NC, TX, MS	Birkenheuer et al. 2004, Holman et al. 2006, Yeagley et al. 2009
		Raccoon	FL	Clark et al. 2012
		Cotton rats	FL	Clark et al. 2012

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Linguatula serrata	Pentastomida	Eastern cottontail rabbit	MS, GA, AL	Andrews and Davidson 1980
Cryptosporidium spp.	Protozoa	Gray fox	NC	Davidson et al. 1992
		Black bear	VA	Duncan et al. 1999
		Domestic dog	FL	Tupler et al. 2012
Cystoisospora spp.	Protozoa	Domestic dog	FL	Tupler et al. 2012
Eimeria spp.	Protozoa	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Giardia spp.	Protozoa	Domestic dog	FL	Tupler et al. 2012
Hepatozoon spp.	Protozoa	Opossum	GA	Kocan et al. 2000, Allen et al. 2011
		Bobcat	GA	Allen et al. 2011
		Domestic cat	OK	Allen et al. 2011
		Coyote	OK, TX	Davis et al. 1978, Allen et al. 2011, Starkey 2013
		Eastern gray squirrel	GA	Allen et al. 2010
		Eastern cottontail rabbit	OK	Allen et al. 2011
		Raccoon	OK	Allen et al. 2011
		Gray fox	GA	Allen et al. 2011
		Mice	OK, GA	Allen et al. 2011
		Woodchuck	MO	Allen et al. 2011
Hepatozoon americanum	Protozoa	Coyote	OK, TX	Kocan et al. 2000, Starkey 2013
		Domestic dog	SE	Gaunt et al. 1983, Barton et al. 1985, Gosset et al. 1985, Mcintire et al. 1997, Panciera et al. 1997, Ewing et al. 2003
Hepatozoon griseisciuri	Protozoa	Gray squirrel	SE	Davidson 1979
Leishmania spp.	Protozoa	Gray fox	NC	Rosypal et al. 2010
		Domestic dog	TN, SC, GA, TX, AL	Grosjean et al. 2003, Duprey et al. 2006

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Neospora caninum	Protozoa	Raccoon	PA, FL	Lindsay et al. 2001
		Domestic dog	SE	Dubey and Lindsay 1996, Lindsay and Dubey 2000
		Coyote	TX	Lindsay et al. 1996
		White-tailed deer	SE	Lindsay et al. 2002
Sarcocystis spp.	Protozoa	Bobcat	FL, WV, GA	Watson et al. 1981, Anderson et al. 1992
		FL panther	FL	Greiner et al. 1989
		Coyote	GA	Holtzman et al. 1992
		Black bear	VA, WV, NC	Crum et al. 1978
		Eastern cottontail rabbit	SE	Andrews and Davidson 1980
		Opossum	SE	Dubey 2000, Baird et al. 2002
Sarcocystis neurona	Protozoa	Raccoon	VA	Hancock et al. 2004
		Domestic cat	VA, PN	Hsu et al. 2010
Toxoplasma gondii	Protozoa	Gray fox	NC, VA	Davidson et al. 1992, Kelly and Sleeman 2003
		Black bear	NC, FL	Dunbar et al. 1998, Nutter et al. 1998
		Coyote	GA, TX	Holtzman et al. 1992, Lindsay et al. 1996
		Red fox	GA	Little et al. 1998
		FL panther	FL	Roekle et al. 1993
		Feral and domestic pigs	NC, GA	Saaverda and Ortega 2004, Sandfoss et al. 2011
		Feral and domestic cats	SE	Vollaire et al. 2005, Nutter et al. 2004
		Raccoon	PA, FL	Lindsay et al. 2001
Trypanosoma actenoides	Protozoa	White-tailed deer	AK	Prestwood 1971
Trypanosoma cruzi	Protozoa	Raccoon	SE	McKeever et al. 1958, Pung et al. 1991, Karsten et al. 1992, Yabsely and Noblet 2002, Brown et al. 2010, Maloney et al. 2010

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
		Opossum	SE	McKeever et al. 1958, Barr et al. 1991, Pung et al. 1991, Karsten et al. 1992
		Bobcat	GA	Brown et al. 2010
		Coyote	GA, VA	Brown et al. 2010
		Striped skunk	GA, FL	McKeever et al. 1958, Brown et al. 2010
		Gray fox	GA, FL, NC, SC	McKeever et al. 1958, Rosypal et al. 2007, Rosypal et al. 2010
		Domestic dog	SE	Meurs et al., 1998, Bradley et al. 2000, Rowland et al. 2010
		Nine-banded armadillo	LA	Yaeger et al. 1988, Barr et al. 1991
		Red fox	VA, NC	Rosypal et al. 2010
Alaria marcinae	Trematode	Black bear	FL	Foster et al. 2004
Alaria mustelae	Trematode	Mink	FL	Foster et al. 2007
Apophallus venustus	Trematode	Raccoon	SE	Harkema and Miller 1964
Ascocotyle pachycystis	Trematode	Raccoon	FL	Schroeder and Leigh 1965
Ascocotyle ampullacea	Trematode	Raccoon	NC	Harkema and Miller 1964
Ascocotyle leighi	Trematode	Raccoon	SC	Harkema and Miller 1964
Atriotaenia procyonis	Trematode	Raccoon	GA	Jordan and Hayes 1959
Baschkirovitrema incrassatum	Trematode	Mink	FL	Foster et al. 2007
Brachylaima Virginianus	Trematode	Black bear	FL	Foster et al. 2004
Carneophallus turgidus	Trematode	Raccoon	NC,SC, GA	Jordan and Hayes 1959, Harkema and Miller 1964
Euparyphium beaveri	Trematode	Raccoon	NC,SC, GA, TN	Harkema and Miller 1964, Bafundo et al. 1980
Euryhelmis squamula	Trematode	Raccoon	NC, TN	Harkema and Miller 1964, Bafundo et al. 1980
Eurytrema procyonis	Trematode	Raccoon	NC, VA, TN	Harkema and Miller 1964, Bafundo et al. 1980

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Fibricola cratera	Trematode	Raccoon	NC, SC, FL	Harkema and Miller 1964
Fibricola texensis	Trematode	Raccoon	TN	Bafundo et al. 1980
Grysoma singularis	Trematode	Raccoon	SC, GA	Harkema and Miller 1964
Gyinaecotyla adunca	Trematode	Raccoon	NC,SC	Harkema and Miller 1964
Hasstilesia tricolor	Trematode	Eastern cottontail rabbit	SE	Andrews and Davidson 1980
Lyperosomum sinuosum	Trematode	Raccoon	SC, TN	Harkema and Miller 1964, Bafundo et al. 1980
Maritreminoides nettae	Trematode	Raccoon	NC,SC, GA	Harkema and Miller 1964
Metagonimoides oregonensis	Trematode	Raccoon	NC, TN	Harkema and Miller 1964, Bafundo et al. 1980
Paragonimus kellicotti	Trematode	Gray fox	WV	Davidson et al. 1992
		Bobcat	WV, GA	Jordan and Byrd 1958, Watson et al. 1981
Paragonimus rudis	Trematode	Raccoon	NC, GA	Harkema and Miller 1964
Parallelorchis diglossus	Trematode	Raccoon	FL, TN	Harkema and Miller 1964, Bafundo et al. 1980
Parametorchis complexus	Trematode	Raccoon	NC	Harkema and Miller 1964
Phagicola diminuta	Trematode	Raccoon	NC,SC	Harkema and Miller 1964
Phagicola longa	Trematode	Raccoon	NC,SC	Harkema and Miller 1964
Pharyngostomoides procyosnis	Trematode	Raccoon	SE	Harkema and Miller 1964, Bafundo et al. 1980
		Black bear	SE	Pence et al. 1983
Procyotrema marsupiformis	Trematode	Raccoon	NC	Harkema and Miller 1964
Prosthodendrium navicsulumn	Trematode	Raccoon	GA	Harkema and Miller 1964
Sellacotyle mustelae	Trematode	Raccoon	NC,SC, GA,VA	Harkema and Miller 1964

(Table C10 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Heterobilharzia americana	Trematode	Raccoon	SE	Harkema and Miller 1964, Bartsch and Ward 1976, Schaffer et al. 1981, Byrd et al. 1967, Schaffer et al. 1981, McKown et al. 1991
		Nine-banded armadillo	LA	Krotoski et al. 1984
		Beaver	TX	Fedynich et al.1986
		Bobcat	LA	Shoop and Corkum 1982
		Coyote	LA, TX	Custer and Pence 1981
		Domestic dog	LA, TX	Malek et al. 1961, Lee 1962, Flowers et al. 2002
		FL panther	FL	Forrester et al. 1985
		Mink	LA	Shoop and Corkum 1982
		Nutria	LA	Malek et al. 1961, Lee 1962
		Opossum	LA	Kaplan 1964
		Swamp rabbit	LA	Malek et al. 1961
		White-tailed deer	SC	Byrd et al. 1967

[1]SE indicates a pathogen was detected across the southeastern United States.

Table C11. Detected disease occurrence in wild North American canids, including bacteria, endoparasites, fungal, and virus infections.

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Brucella spp	Bacteria	San Joaquin kit fox	CA	McCue and O'Farrel 1988
Ehrlichia spp.	Bacteria	Coyote	CA	Pusterla et al. 2000
	Bacteria	Coyote	OK, TX	Starkey et al. 2013
Francisella tularensis	Bacteria	Coyote	UT	Arjo et al. 2003
	Bacteria	Coyote	NE	Biscof and Rogers 2005
	Bacteria	Coyote	YNP	Gese et al. 1997
	Bacteria	Coyote	CO	Gese et al. 2004
	Bacteria	San Joaquin kit fox	CA	McCue and O'Farrel 1988
Rickettsia spp.	Bacteria	Coyote	NE	Biscof and Rogers 2005
	Bacteria	Coyote	OK, TX	Starkey et al. 2013
Yersinia pestis	Bacteria	Coyote	UT	Arjo et al. 2003
	Bacteria	Coyote	CO	Gese et al. 2004
	Bacteria	Coyote	YNP	Gese et al. 1997
	Bacteria	Swift foxes	CO	Gese et al. 2004
Neospora caninum	Coccidia	Gray wolf	YNP	Almberg et al. 2009
Blastomyces dermatitidis	Fungus	Gray wolf	Ontario, Canada	Krizan 2000, Paquet et al. 2002
Leishmania spp	Protozoan	Coyote	PN	Rosypal et al. 2013
	Protozoan	Red fox	PN	Rosypal et al. 2013
Cache Valley Virus	Virus	Swift foxes	CO, CA, NM, AZ	Miller et al. 2000
	Virus	Kit fox	CO, CA, NM, AZ	Miller et al. 2000
Canine adenovirus type-1	Virus	Gray wolf	YNP	Almberg et al. 2009
	Virus	Coyote	YNP	Almberg et al. 2009
	Virus	Coyote	UT	Arjo et al. 2003
	Virus	Coyote	CA	Cypher et al. 1998
	Virus	Coyote	CO	Gese et al. 2004
	Virus	Red fox	YNP	Almberg et al. 2009

(Table C11 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
Canine coronavirus	Virus	Coyote	TX, UT, WA , CO	Foreyt and Evermann 1985
Canine distemper virus	Virus	Gray wolf	YNP	Almberg et al. 2009
	Virus	Gray wolf	Manitoba, Canada	Carbyn 1982
	Virus	Gray wolf	MT	Johnson et al. 1994
	Virus	Gray wolf	Canadian Rockies	Nelson et al. 2012
	Virus	Coyote	YNP	Gese et al. 1997, Almberg et al. 2009
	Virus	Coyote	UT	Arjo et al. 2003
	Virus	Coyote	NE	Biscof and Rogers 2005
	Virus	Coyote	CA	Cypher et al. 1998
	Virus	Coyote	CO	Gese et al. 1991, Gese et al. 2004
	Virus	Red fox	YNP	Almberg et al. 2009
	Virus	Swift foxes	SW	Miller et al. 2000, Gese et al. 2004
	Virus	Kit fox	SW	Miller et al. 2000
	Virus	San Joaquin kit fox	CA	McCue and O'Farrel 1988
Canine herpesvirus	Virus	Gray wolf	YNP	Almberg et al. 2009
	Virus	Coyote	YNP	Almberg et al. 2009
	Virus	Red fox	YNP	Almberg et al. 2009
Canine infectious hepatitis	Virus	Coyote	YNP	Gese et al. 1997
	Virus	Gray wolf	MT	Johnson et al. 1994
	Virus	San Joaquin kit fox	CA	McCue and O'Farrel 1988
Canine parvovirus	Virus	Gray wolf	YNP	Almberg et al. 2009
	Virus	Gray wolf	MT	Johnson et al. 1994
	Virus	Gray wolf	MN	Mech and Goyal 1993, Mech et al. 2008
	Virus	Gray wolf	Canadian Rockies	Nelson et al. 2012

(Table C11 continued)

Pathogen	Tax. origin	Detected species	Location[1]	Reference
	Virus	Coyote	YNP	Gese et al. 1997, Almberg et al. 2009
	Virus	Coyote	UT	Arjo et al. 2003
	Virus	Coyote	CA	Cypher et al. 1998
	Virus	Coyote	CO	Gese et al. 1998, Gese et al. 2004
	Virus	Red fox	YNP	Almberg et al. 2009
	Virus	Swift foxes	SW	Miller et al. 2000, Gese et al. 2004
	Virus	Kit fox	SW	Miller et al. 2000
	Virus	San Joaquin kit fox	CA	McCue and O'Farrel 1988, Miller et al. 2000
Colorado tick fever	Virus	Swift foxes	CA	Miller et al. 2000
	Virus	Kit fox	CA	Miller et al. 2000
Flaviviruses	Virus	Coyote	NE	Biscof and Rogers 2005
Jamestown Canyon virus	Virus	Swift foxes	CO, NM, AZ	Miller et al. 2000
	Virus	Kit fox	CO, NM, AZ	Miller et al. 2000
Rabies	Virus	Gray wolf	Ontario, Canada	Theberge et al. 1994
Western equine encephalitis	Virus	Swift foxes	CA	Miller et al. 2000
	Virus	Kit fox	CA	Miller et al. 2000
Vesicular stomatitis Indiana	Virus	Swift foxes	SW	Miller et al. 2000
	Virus	Kit fox	SW	Miller et al. 2000

[1]YNP is Yellowstone National Park and SW indicates a pathogen was detected across the southwestern United States.

Table C12. Bacterial pathogens detected in southeastern wildlife populations based on articles containing the words ["United States" AND south* AND (_disease_ OR _parasit*_ OR _pathogen_)] and keyword searches in the following journals: *Journal of Zoo and Wildlife Medicine*, *Journal of Wildlife Disease*, *Journal of Veterinary Medicine, American Journal of Veterinary Research, Journal of Parasitology, American Midland Naturalist*, and *Southeastern Naturalist*.

Pathogen	Detected species	Location[1]	Reference
Anaplasma phagocytophilum	White-tailed deer	NC	Sherrill et al. 2012
Bartonella vinsonii	Domestic dog	SE	Honadel et al. 2001
Borrella burgdorferi	Raccoon	E US	Magnarelli et al. 1991, Oueflette et al. 1997, Ryan et al. 2000
(lyme disease)	White-tailed deer	E US	Magnarelli et al. 1991, Oliver 1996, Sherrill et al. 2012
	House mouse	NC	Ryan et al. 2000
	White-footed mouse	NC	Ryan et al. 2000
	Marsh rice rat	NC	Ryan et al. 2000
	Marsh rabbit	NC	Oliver 1996, Ryan et al. 2000
	Cotton mouse	SC, GA, FL	Oliver 1996, Lin et al. 2004
	Eastern woodrat	SC, FL	Oliver 1996, Lin et al. 2004
	Hispid cotton rat	SC, GA, FL	Oliver et al. 1995, Oliver 1996, Lin et al. 2004
	Eastern cottontail rabbit	MO, GA	Lin et al. 2004
Bronchopneumonia	Gray fox	NC	Davidson et al. 1992
Clostridium perfringens	Domestic dog	FL	Tupler et al. 2012
Ehrlichia spp.	Raccoon	SE	Davidson et al. 1999, Comer et al. 2000,
	White-tailed deer	SE	Lockhart et al. 1995, Little et al. 1998, Yabsley et al. 2003
	Opossum	GA	Lockhart et al. 1997
	Domestic dog	SE	Lockhart et al. 1997
Leptospira	Coyote	SC	Millet et al. 2009
	Domestic dog	SE	Moore et al. 2006
Listeria monocytogene	Gray fox	MS	Black et al. 1996
Salmonella spp	Domestic dog	FL	Tupler et al. 2012
Tick paralysis	Gray fox	GA	Davidson et al. 1992
Yersinia pseudotuberculosis	Gray fox	MS	Black et al. 1996

[1]SE and E US indicates a pathogen was detected across the southeastern and Eastern United States, respectively.

Table C13. Published accounts of suspected or documented disease mediated population declines in threatened species.

Species	Disease[1]	Tax. origin	Location	Population effects	Source	Reference
African wild dogs	CDV or rabies	Virus	Kenya, Africa	Inferred local extinction	Domestic dog	Alexander et al. 1993, Alexander and Appel 1994
	Rabies	Virus	Kenya, Africa	Reduced population	Domestic dog	Kat et al. 1995, Kat et al. 1996
	Rabies	Virus	Tanzania, Africa	Reduced population	Domestic dog	Gascoyne et al. 1993
	Rabies	Virus	South Africa	Reduced population	Domestic dog	Van Heerden et al. 1995, Hofmeyr et al. 2000
	CDV or rabies	Virus	Tanzania, Africa	Reduced population	Domestic dog	Burrows et al. 1994
	Rabies	Virus	Namibia, Africa	Prevented reintroduction	Jackal	Scheepers and Venzke 1995
Arctic fox	Mange	Mite	Medny Island, Russian	Catastrophic cub mortality	Domestic dog	Goltsman et al. 1996,
Black-footed ferret	CDV	Virus	WY, US	Reduced population, extirpated from wild	Badgers/coyotes?	Thorne and Williams 1988, Williams et al. 1988
	Sylvatic plague	Bacteria	WY, MT, US	Influenced reintroductions: indirect by killing obligate prey species	Prairie dogs	Williams et al. 1994, Matchett et al. 2010
Ethiopian wolf	Rabies	Virus	Ethiopia, Africa	Reduced population	Domestic dog	Sillero-Zubiri et al. 1996, Randall et al.2004
Gray wolves	CPV	Virus	MN, US	Increased pup mortality	Domestic dog	Mech et al. 2008

(Table C13 continued)

Species	Disease[1]	Tax. origin	Location	Population effects	Source	Reference
Island fox	CDV	Virus	Santa Catalina Island, CA	Reduced population	Raccoon	Timm et al. 2009
Jackal	CDV	Virus	Kenya, Africa	Reduced population	Domestic dog	Alexander and Appel 1994
Sea otter	Acanthocephala	Worm	CA, US	May limit recovery		Kreuder et al. 2003
	T. gondii	Protozoa	CA, US	May limit recovery		Kreuder et al. 2003
Serengeti lions	CDV	Virus	Tanzania, Africa	Reduced population	Domestic dog	Roelke-Parker et al. 1996

[1]Canine distemper virus (CDV) and Canine parvovirus (CPV)

APPENDIX D: SUPPLEMENTAL FIGURES AND TABLES FROM CHAPTER 5

Table D1. Primers and primer conditions used to amplify toll-like receptor (TLR) and major histocompatibility complex genes in red wolves (*Canis rufus*) and coyotes (*Canis latrans*).

Gene	Exon	Sequence ('5-'3)	PCR conditions	Citation
TLR1	partial exon 1A (F)	GATCTTTACCCGAATTGCGA	95°Cx4",35x(95°Cx60",58°Cx 60",72°Cx120"), 72°Cx10'	House et al. 2009
TLR1	partial exon 1A (R)	AATTTGAGATGGGCAAACCA		House et al. 2009
TLR1	partial exon 1B (F)	ATGCATTCAATTTGCCACAA	95°Cx4",35x(95°Cx60",58°Cx 60",72°Cx120"), 72°Cx10'	House et al. 2009
TLR1	internal SNP (F)	TTCTGCCTGGGTGAAGAGTG	95°Cx4",35x(95°Cx60",52°Cx 60",72°Cx120"), 72°Cx10'	self designed
TLR1	partial exon 1B (R)	GGTGAACTGGAGAGCCTGAA		House et al. 2009
TLR4	full exon 1 (F)	TTCCTCTTGCCCCTTAACTC	95°Cx4",35x(95°Cx60",50°Cx 60",72°Cx120"), 72°Cx10'	Kathrani et al. 2010
TLR4	full exon 1 (R)	GCCATGTAACCATGAACTGT		Kathrani et al. 2010
TLR4	full exon 2 (F)	GATGGATGGATGGACAGACC	94°Cx4",35x(95°Cx60",58°Cx 60",72°Cx120"), 72°Cx10'	Kathrani et al. 2010
TLR4	full exon 2 (R)	TTCACAGATGAGGCAATGGG		Kathrani et al. 2010
TLR4	partial exon 3 (F)	GGTCTGGCTGGCTTAAAG	95°Cx4",30x(95°Cx60",50°Cx 60",72°Cx180"), 72°Cx10'	Kathrani et al. 2010
TLR4	internal SNP exon 3(F)	TCCCTCAGAAACCTCCGT	95°Cx4",35x(95°Cx60",51°Cx 60",72°Cx120"), 72°Cx10'	self designed
TLR4	partial exon 3 (R)	CAACTTCCACCAAGAGCT		Kathrani et al. 2010
TLR5	partial exon 1 (F)	GATCTCCTCCTTGGTGCTCG	94°Cx4",35x(95°Cx60",60°Cx 60",72°Cx120"), 72°Cx10'	self designed
TLR5	partial exon 1 (R)	TGTTGTGCGTTAGGTCCACG		self designed
TLR6	partial exon 1A (F)	CAACAACCCTTTGGGGAATA	95°Cx4",30x(95°Cx60",50°Cx 60",72°Cx180"), 72°Cx10'	House et al. 2009
TLR6	partial exon 1A (R)	CACCTTGACCTTGGGAGGTA		House et al. 2009

(Table D1 continued)

Gene Exon	Sequence ('5-'3)	PCR conditions	Citation
TLR6 partial exon 1B (F)	TGCACTTGGGTTGGGAGTAT	95°Cx4",30x(95°Cx60",55°Cx 60",72°Cx180"), 72°Cx10'	House et al. 2009
TLR6 partial exon 1B (R)	TCTGCGTTATTGTTTTCAGCA		House et al. 2009
DRB exon 2 (F)	CCGTCCCCACAGCACATTTC	95°Cx4',40x(95°Cx30",57°Cx 60",72°Cx60"), 72°Cx10'	Wagner et al. 1996, Wagner et al. 1998, Hedrick et al. 2002, Kennedy et al. 2007
DRB exon 2 (R)	TGTGTCACACACCTCAGCACCA		Wagner et al. 1996, Wagner et al. 1998, Hedrick et al. 2002, Seddon and Ellegren 2002
DQA exon 2 (F)	CTCAGCTGACCATGTTGC	95°Cx4',40x(95°Cx30",62°Cx 60",72°Cx60"), 72°Cx10'	Kennedy et al. 2004, Wilbe et al. 2009
DQA exon 2 (R)	GGACAGATTCAGTGAAGAGA		Wagner et al. 1996, Seddon and Ellegren 2002, Kennedy et al. 2007, Wilbe et al. 2009
DQB exon 2 (F)	TCACTGGCCCGGCTGTCTCC	95°Cx4',15x(95°Cx40",73°-0.5°C each cycle x 60",72°Cx60"),	Wagner et al. 1998, Seddon and Ellegren 2002
DQB exon 2 (R)	GGTGCGCTCACCTCGCCGCT	25x(95°Cx40",66°x60",72°Cx60"), 72°Cx10'	Wagner et al. 1998, Seddon and Ellegren 2002

Table D2. The number of individual red wolves (*Canis rufus*) and coyotes (*Canis latrans*) with DNA sequences for each parasite and immune measure (bactericidal killing assays (BKA), complete blood counts (CBC)).

	Red wolf	Coyote	Total
BKA	35	32	67
CBC	35	17	52
Heartworm	37	30	67
Tick-borne assays	30	27	57
Endoparasites	32	17	49

Table D3. Toll-like receptor (TLR) and major histocompatibility complex (MHC) heterozygosity assocations with immune measures (*E. coli* and *C. albicans* bacteria killing capacity, white blood cell to red blood cell ratio (WBC:RBC)) and disease measures (endoparasite loads, heartworm infection, Ehrlichia and canine parvovirus (CPV) exposure) in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). (-) and (+) indicate immune gene heterozygosity was in the top generalized linear mixed effect model set; (-) means heterozygosity was negatively association with immune ability or disease prevalence, (+) means heterozygosity was positively associated with immune ability or disease prevalence. Highlighted samples have 95% confidence limits not overlapping 0.

	TLR4ex1	TLR4ex2*	TLR4ex3	TLR5	TLR6	MHC	DRB	DQB	DQA
BKA *E. coli*	-	-	-	-	-	-	-	-	-
BKA *C. albican*	-	-	+	+	-	-	-	-	+
WBC:RBC			-	-	-			-	
Endoparasites	+	+	+	-	+	+		+	+
Heartworm	+	+	+	-	-		+	-	-
Ehrlichia	+	+	+	-	-	-		-	-
CPV	-	-	-	-	-		-	-	-

*TLR4ex2: only 3 heterozygotes total

Table D4. Toll-like receptor (TLR) haplotype and major histocompatibility complex (MHC) allele associations with *E. coli* and *C. albicans* bacteria killing capacity, white blood cell to red blood cell ratio (WBC:RBC), and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). (*) indicate TLR or MHC allele variables were in the top generalized linear mixed effect model set; highlighted samples have 95% CL not overlapping 0.

	TLR4ex1	TLR4ex2	TLR4ex3	TLR5	TLR6	DRB1	DQB1	DQA
BKA *E. coli*	*	*	*	*	*	*	*	*
BKA *C. albican*	*	*	*	*	*	*	*	*
WBC:RBC		*	*	*	*			
Endoparasites		*	*	*	*		*	*

Table D5. Toll-like receptor (TLR) and major histocompatibility complex (MHC) allele associations with heartworm infection and Ehrlichia and canine parvovirus (CPV) exposure in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Results derived from Fisher exact tests; bold samples have *P*<=005.

	TLR4ex1	TLR4ex2	TLR4ex3	TLR5	TLR6	DRB1	DQB1	DQA
Heartworm	NS	NS	NS	NS	NS	NS	NS	NS
Ehrlichia	NS	NS	NS	NS	SGF	NS	NS	NS
CPV	NS	NS	SGF	NS	NS	NS	NS	NS

Table D6. Toll-like receptor (TLR) and major histocompatibility complex (MHC) SNP associations with immune measures (*E. coli* and *C. albicans* bacteria killing capacity, white blood cell to red blood cell ratio (WBC:RBC)) and disease measures (endoparasite loads, heartworm infection, Ehrlichia and canine parvovirus (CPV) exposure) in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). (-) and (+) indicate the immune gene variable for SNPs was in the top generalized linear mixed effect model set; (-) means SNP number was negatively association with immune ability or disease prevalence, (+) means SNP number was positively associated with immune ability or disease prevalence. Highlighted samples have 95% confidence limits not overlapping 0. TLR4 SNP numbers were the same as heterozygosity (Y/N) and thus are not presented again.

	TLR5	TLR6	DRB	DQB	DQA
BKA *E. coli*	+	-	-		+
BKA *C. albican*	-	+	-		-
WBC:RBC	-	-			
Endoparasites	+	+		+	
Heartworm	-	-			+
Ehrlichia	-	+	-	-	-
CPV	+	+	+	-	-

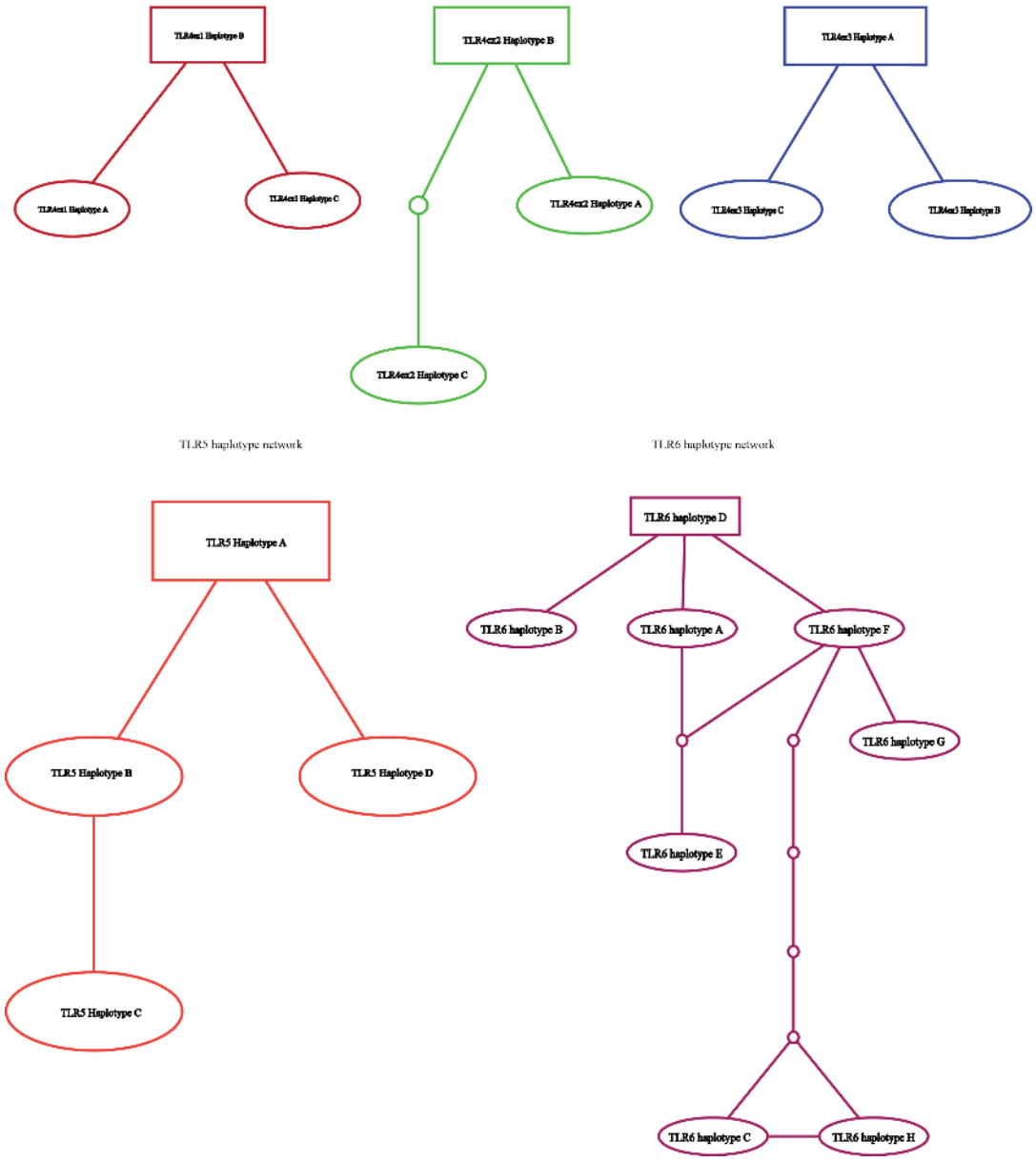

Figure D1. Toll-like receptor haplotype networks built from amino acid matrixes in TCS 1.21 assessing functional similarity in TLR4, TLR5, and TLR6 gene sequences from red wolf (*Canis rufus*) and coyotes (*Canis latrans*). All TLR4 exon haplotypes correspond to nucleotide alleles of same name in Table 1. TLR 5 Haplotype A encompasses synonymous nucleotide alleles 5a, 5b, 5c, 5f, 5h, 5k, 5m; TLR5 Haplotype B encompasses synonymous nucleotide alleles 5d, 5e, 5j; TLR5 Haplotype C is nucleotide allele 5i; TLR5 Haplotype D is allele 5g. TLR 6 Haplotype A encompasses synonymous nucleotide alleles 6a, 6d, 6g, 6h, 6m, 6n, 6o; TLR 6 Haplotype B encompasses nucleotide allele 6b; TLR 6 Haplotype C encompasses nucleotide allele 6f; TLR 6 Haplotype D encompasses synonymous nucleotide alleles 6e, 6q; TLR 6 Haplotype E encompasses nucleotide alleles 6j; TLR 6 Haplotype F encompasses synonymous nucleotide alleles 6i, 6k; TLR 6 Haplotype G encompasses synonymous nucleotide alleles 6c, 6p; TLR 6 Haplotype H encompasses nucleotide alleles 6l.

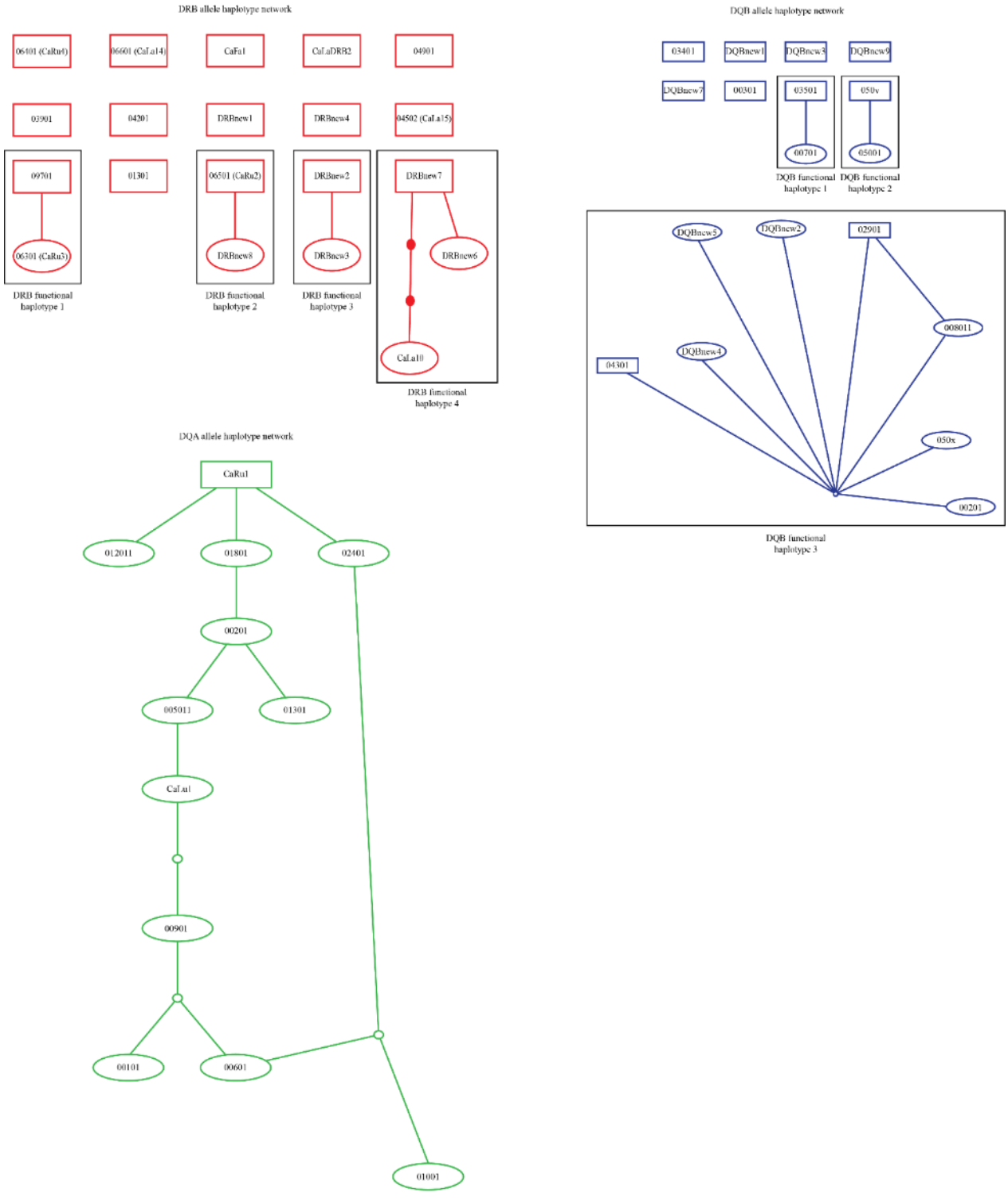

Figure D2. Major histocompatibility complex (MHC) allele haplotype networks built from amino acid matrixes in TCS 1.21 assessing functional similarity in DRB, DQB, and DQA gene sequences from red wolf (*Canis rufus*) and coyotes (*Canis latrans*).

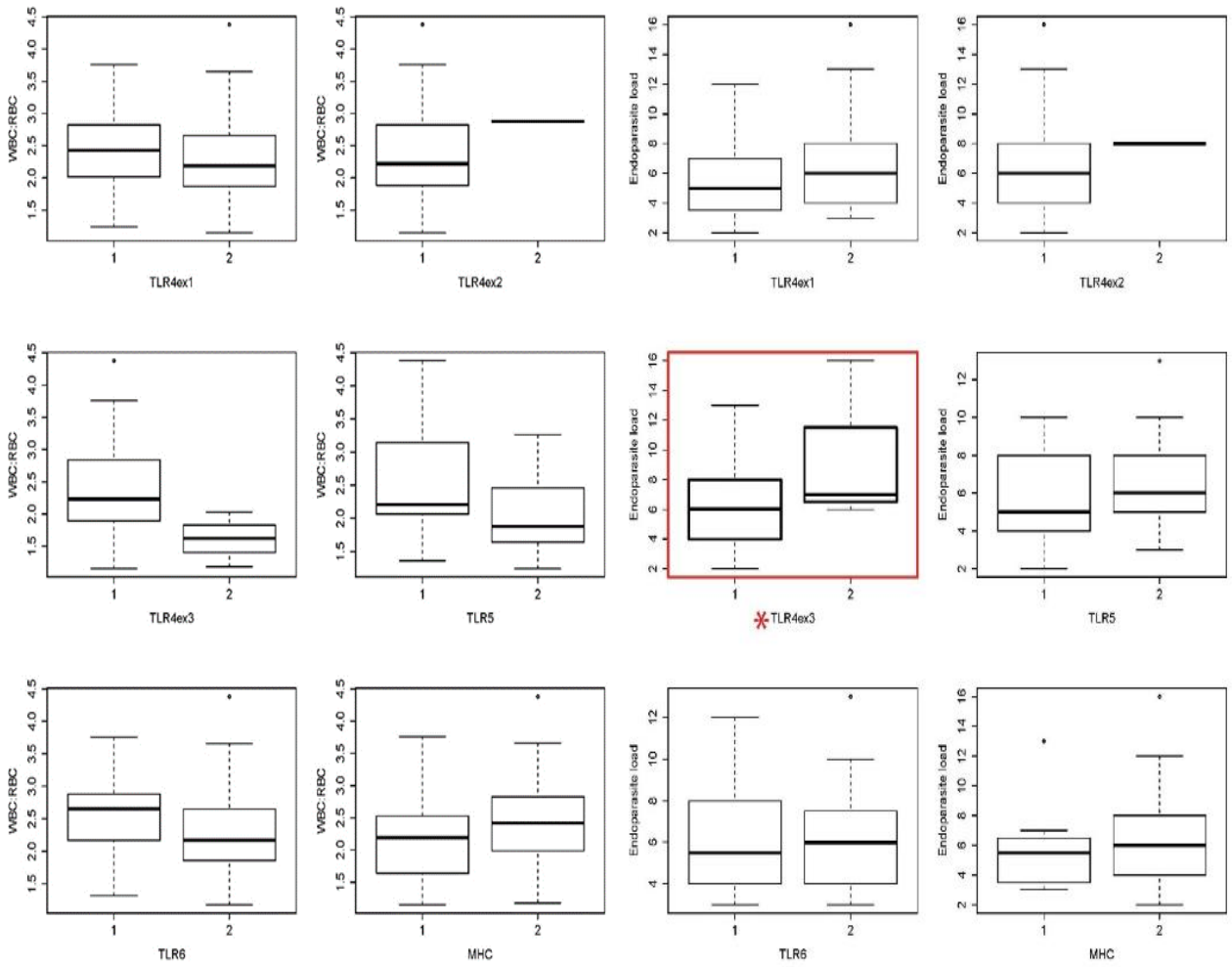

Figure D3. Box-and-whisker plots comparing the number of homozygotes (1) and heterozygotes (2) associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 heterozygotes had significantly poorer killing capacity in *E. coli* assays and higher endoparasite loads.

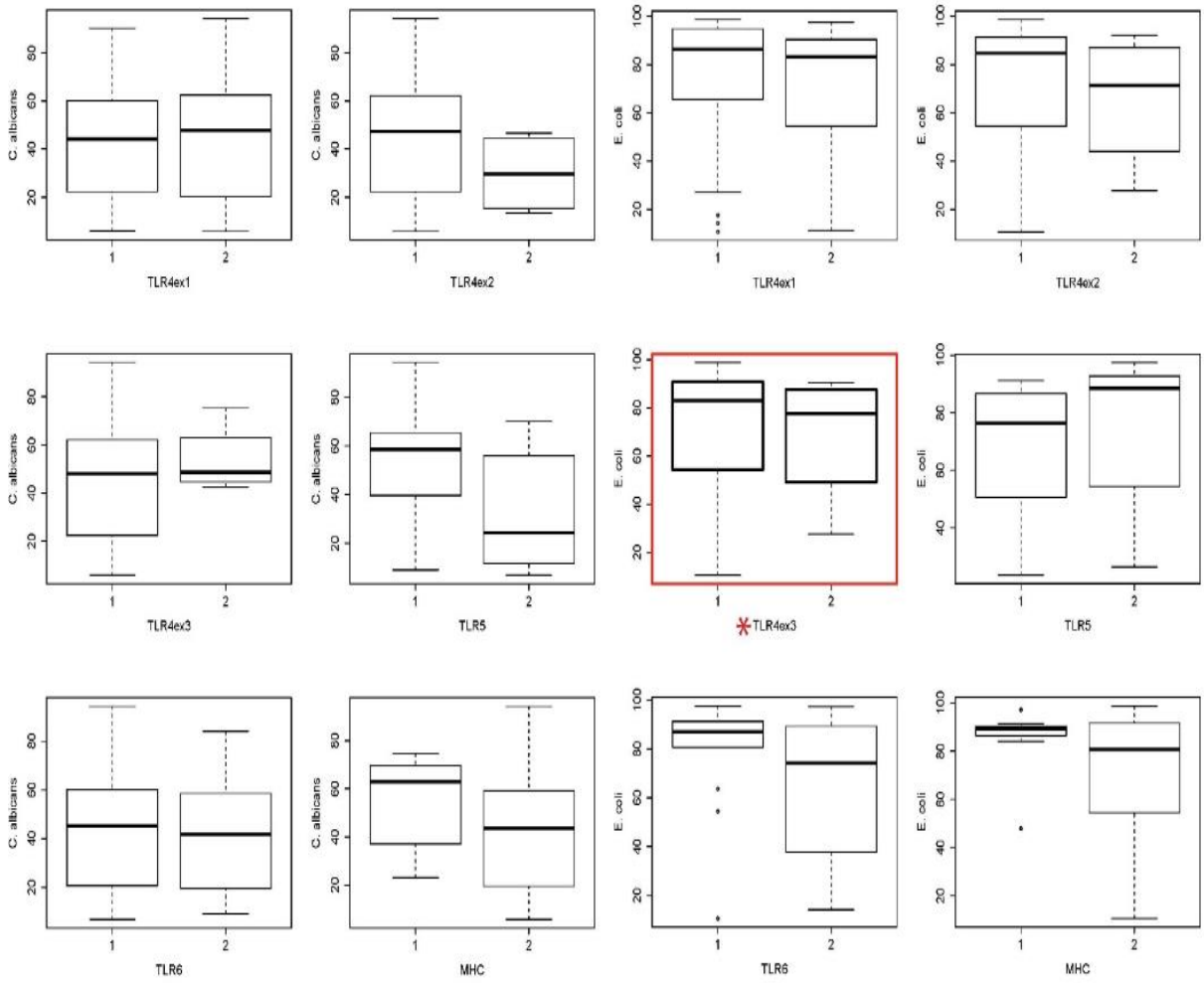

Figure D3 continued. Box-and-whisker plots comparing the number of homozygotes (1) and heterozygotes (2) associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 heterozygotes had significantly poorer killing capacity in *E. coli* assays and higher endoparasite loads.

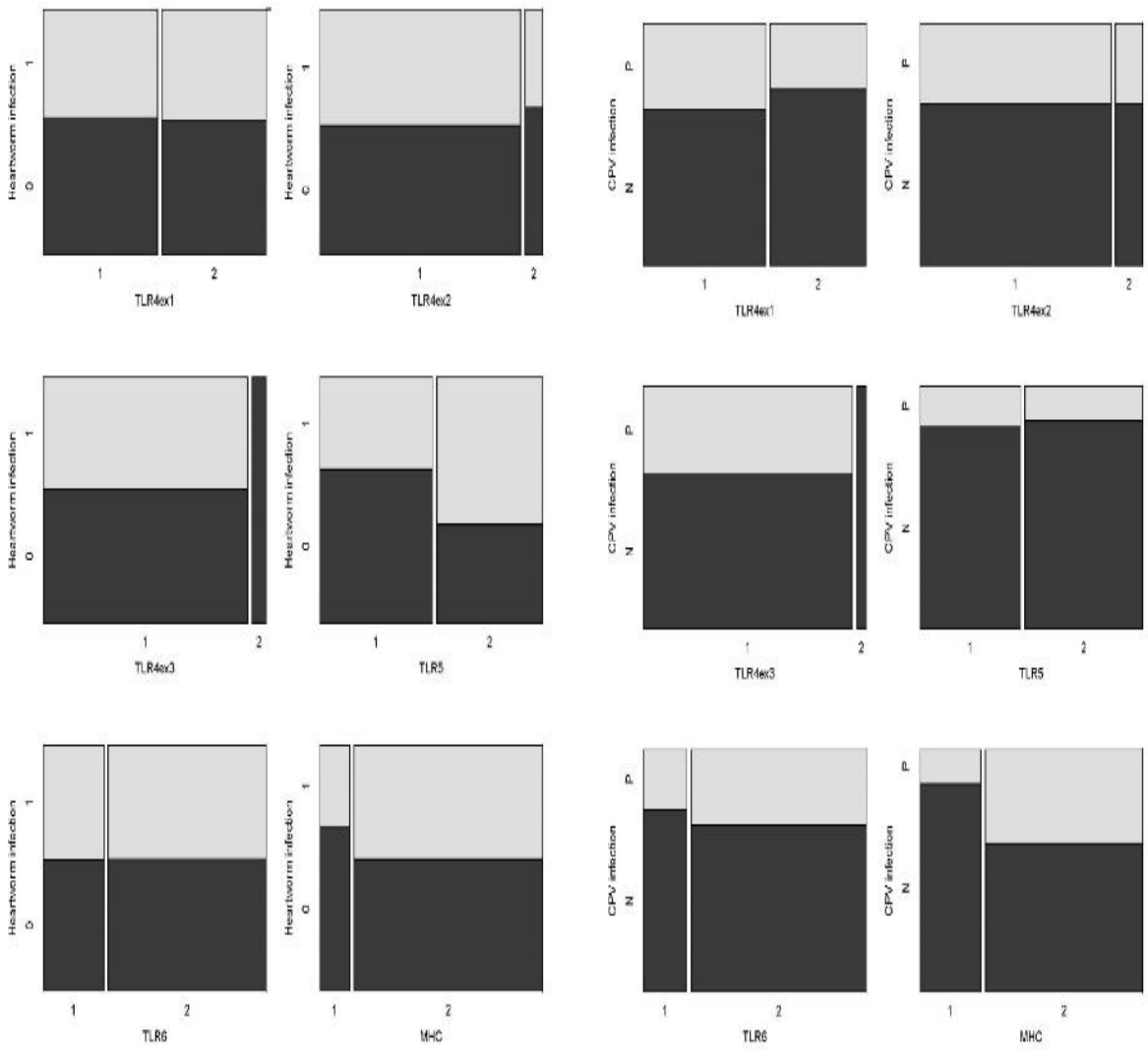

Figure D4. Bar plots comparing the number of homozygotes (1) and heterozygotes (2) with prevalence of heartworm infection (negative=0, positive=1) and canine parvovirus (CPV) exposures (exposure titers negative=0, exposure titers positive=1) in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes.

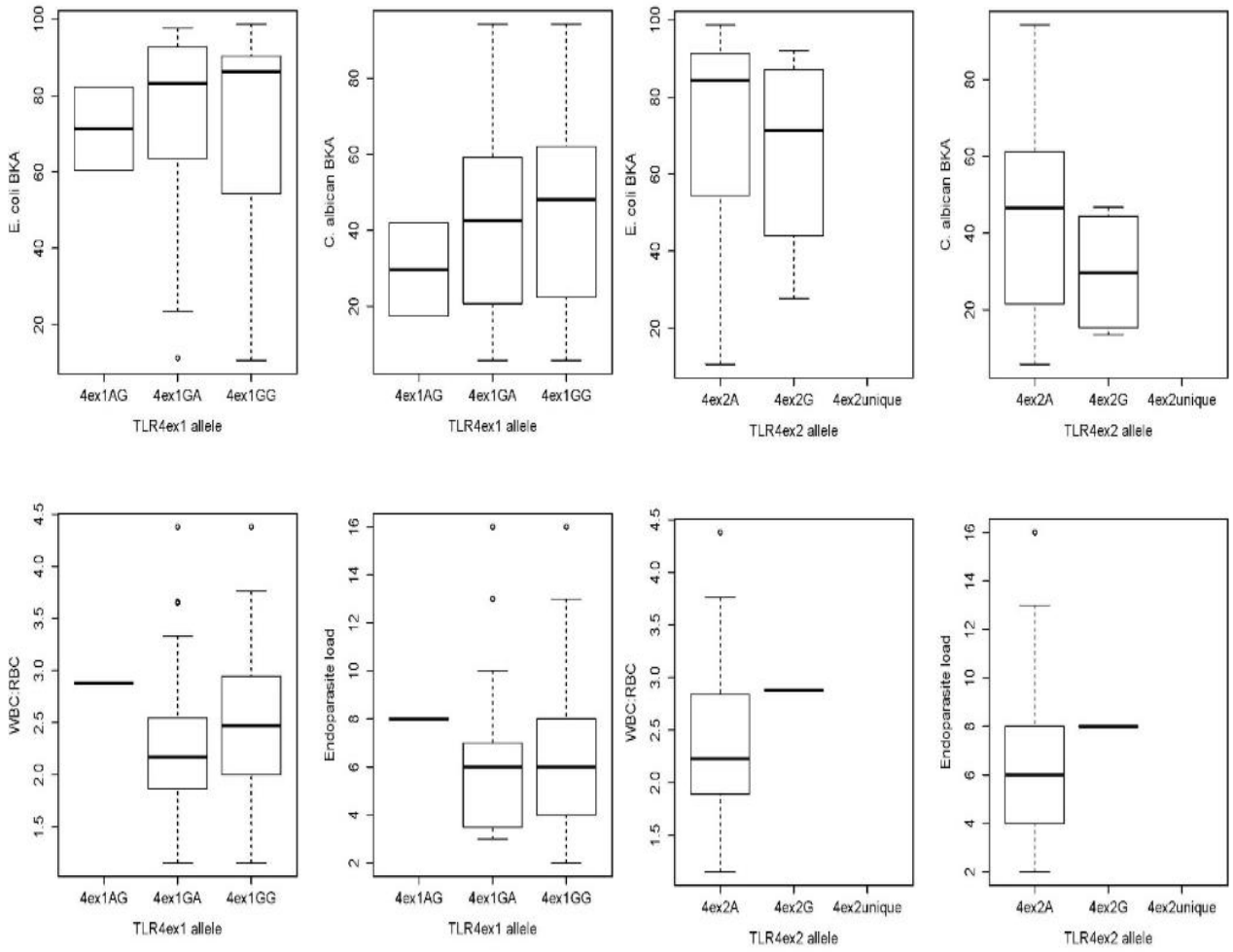

Figure D5. Box-and-whisker plots comparing the alleles of innate immune genes TLR4, TLR5, TLR6 and adaptive immune genes MHC DRB, DQB, and DQA associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher endoparasite loads.

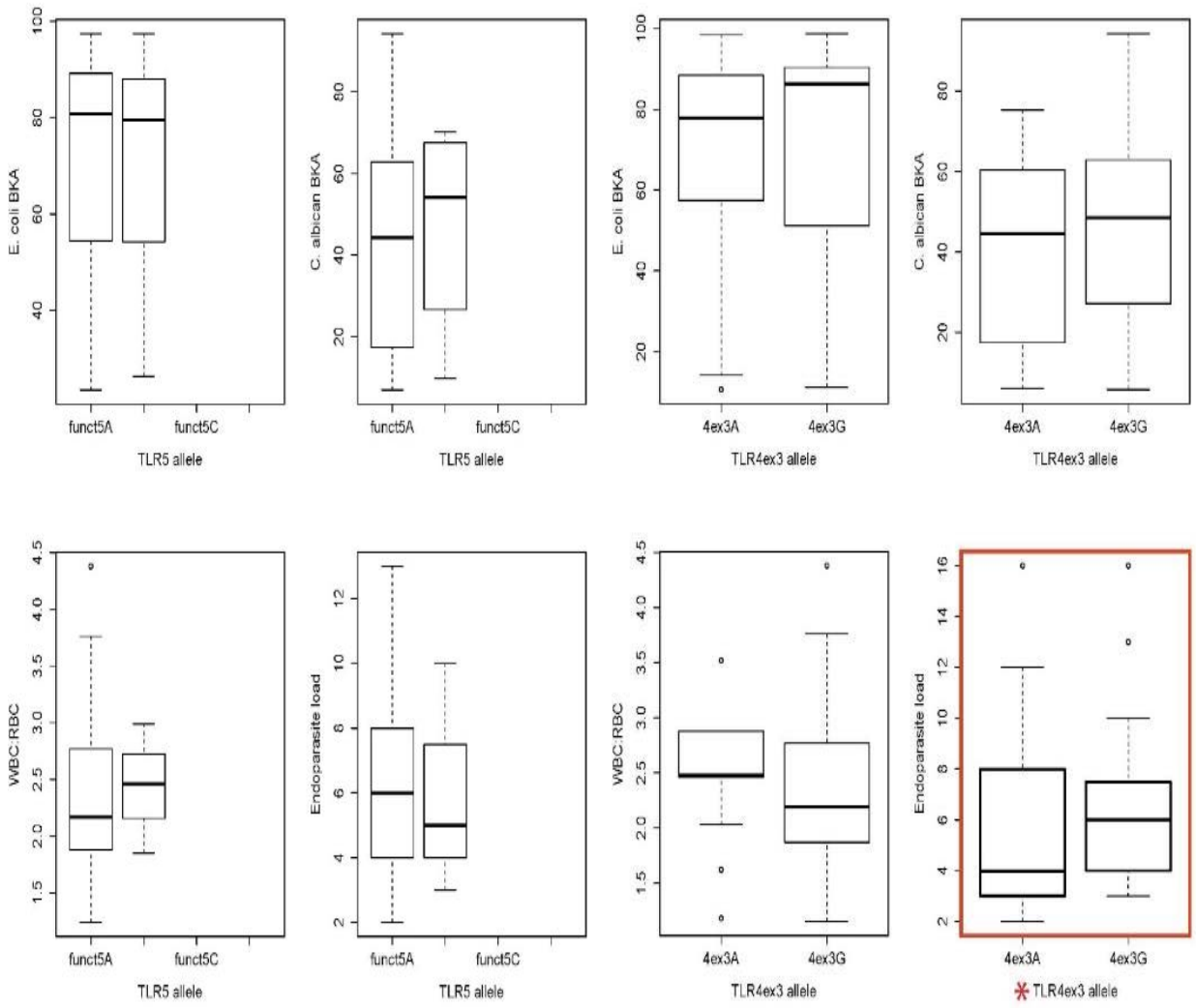

Figure D5 continued. Box-and-whisker plots comparing the alleles of innate immune genes TLR4, TLR5, TLR6 and adaptive immune genes MHC DRB, DQB, and DQA associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher endoparasite loads.

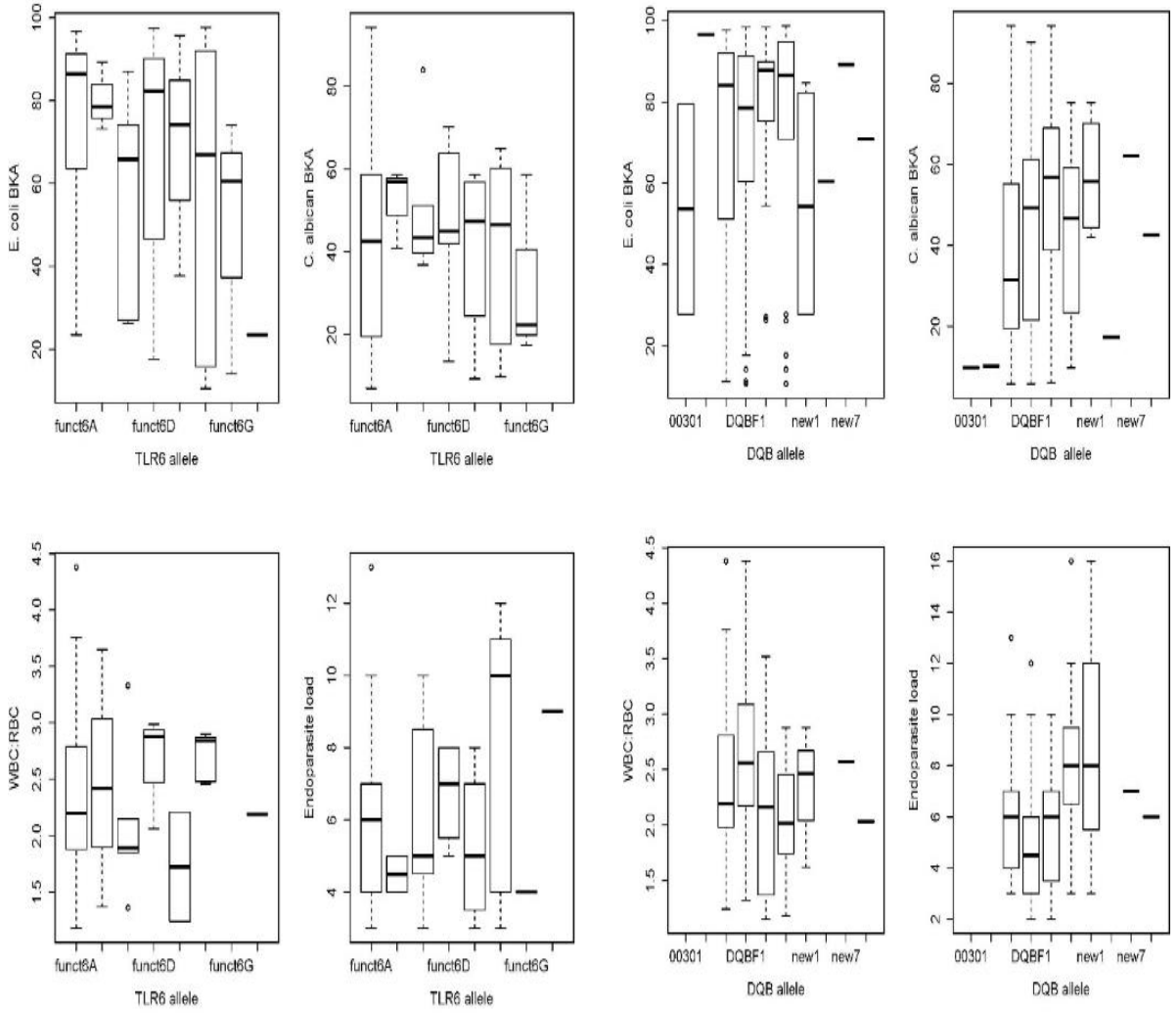

Figure D5 continued. Box-and-whisker plots comparing the alleles of innate immune genes TLR4, TLR5, TLR6 and adaptive immune genes MHC DRB, DQB, and DQA associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher endoparasite loads.

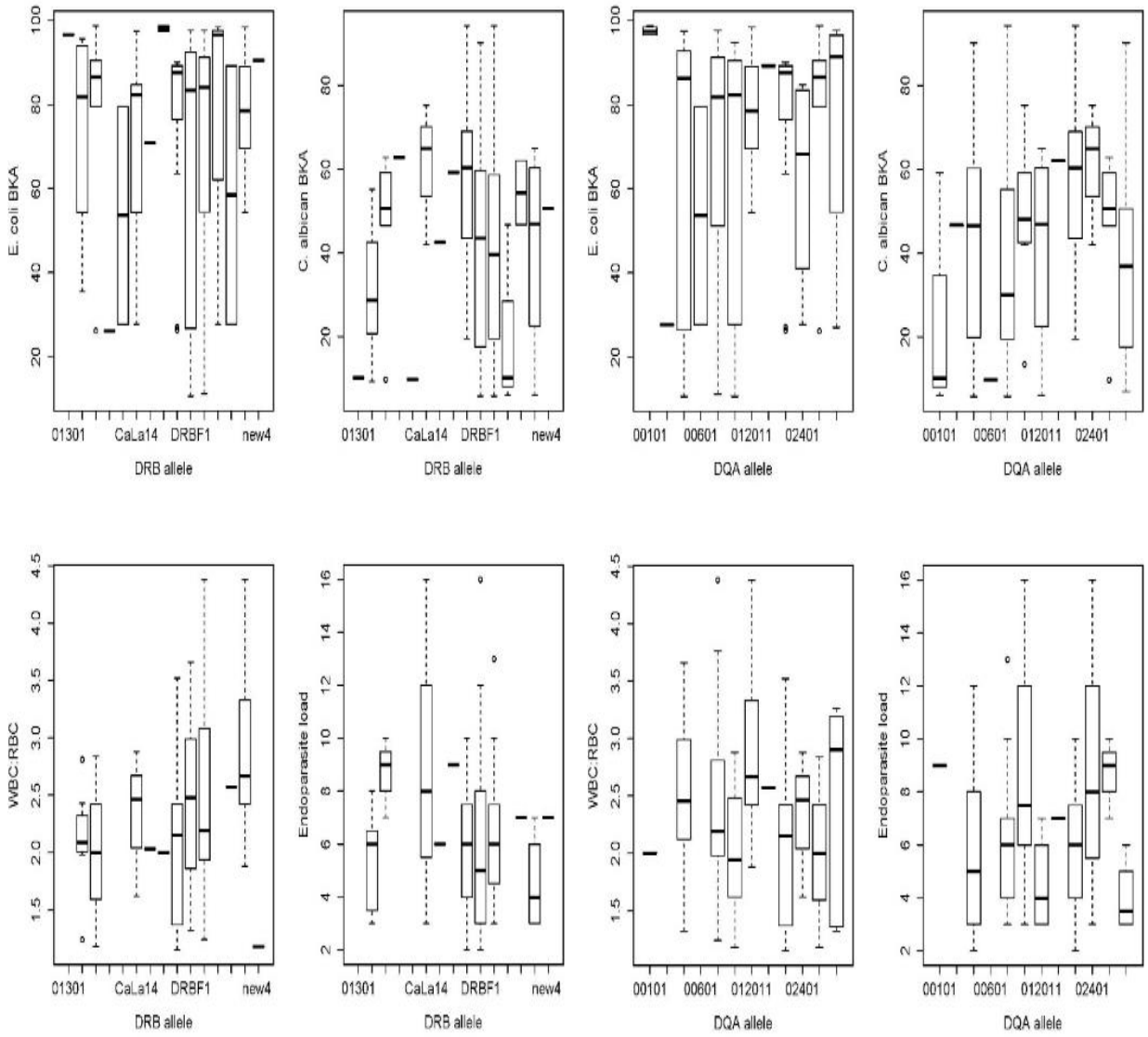

Figure D5 continued. Box-and-whisker plots comparing the alleles of innate immune genes TLR4, TLR5, TLR6 and adaptive immune genes MHC DRB, DQB, and DQA associated with immune measures (*E. coli* and C. *albicans* bacteria killing capacity, and white blood cell to red blood cell ratio (WBC:RBC)) and endoparasite loads in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher endoparasite loads.

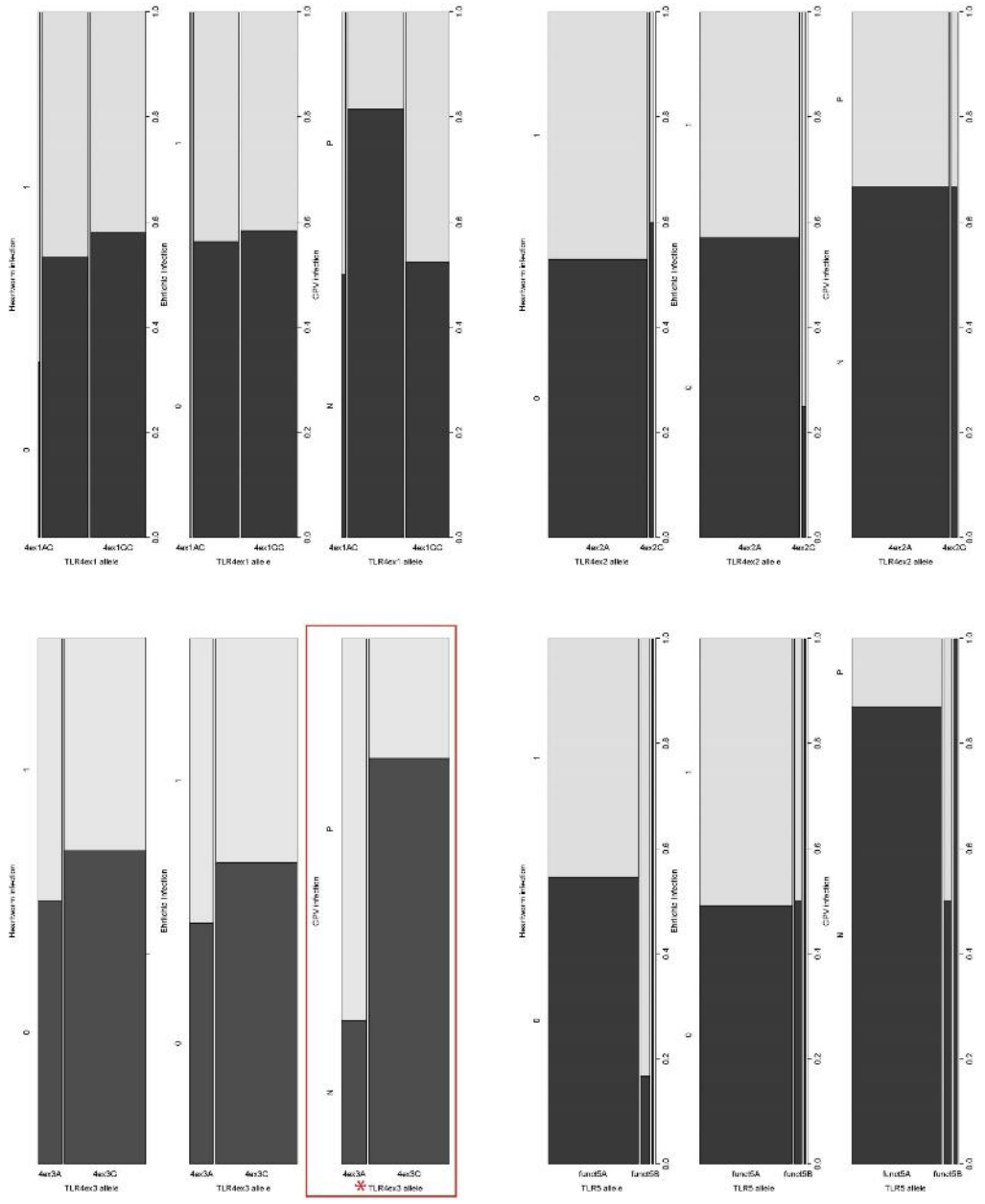

Figure D6. Bar plots comparing alleles of innate immune TLR4, TLR5, TLR6 genes and adaptive immune MHC DRB, DQB, and DQA genes associated with prevalence of heartworm infection (negative=0, positive=1) and canine parvovirus (CPV) exposures (exposure titers negative=0, exposure titers positive=1) in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher CPV exposure rates; several highlight TL6 haplotypes were marginally significant (*P*=0.050).

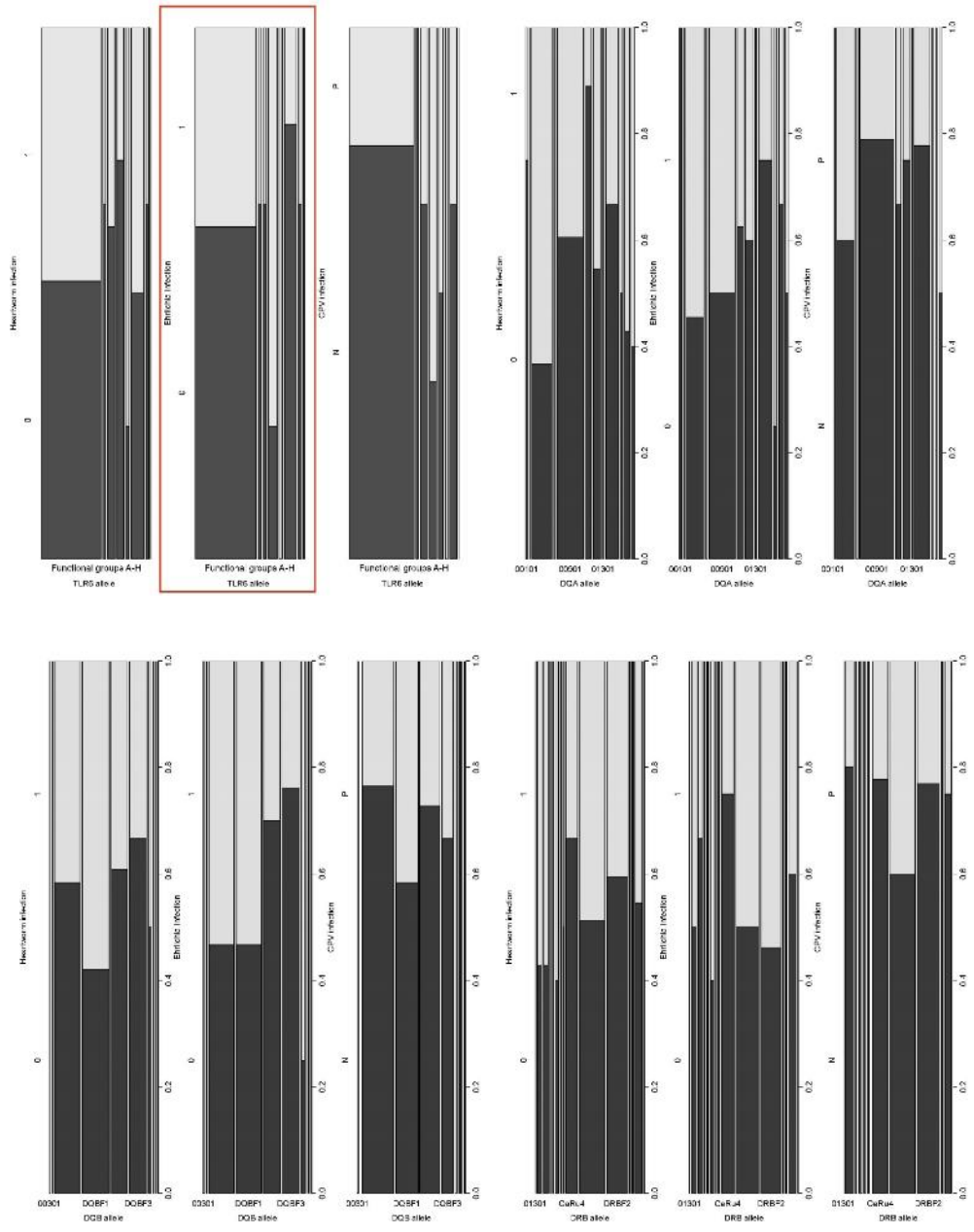

Figure D6 continued. Bar plots comparing alleles of innate immune TLR4, TLR5, TLR6 genes and adaptive immune MHC DRB, DQB, and DQA genes associated with prevalence of heartworm infection (negative=0, positive=1) and canine parvovirus (CPV) exposures (exposure titers negative=0, exposure titers positive=1) in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). The bottom of the box is the 25th percentile, the top is the 75th, the middle line represents the median value, and whiskers extend to the highest and lowest observation homozygotes and heterozygotes. Significant associations are highlighted in red, where TLR4 exon 3 haplotype G had significantly higher CPV exposure rates; several highlight TL6 haplotypes were marginally significant (P=0.050).

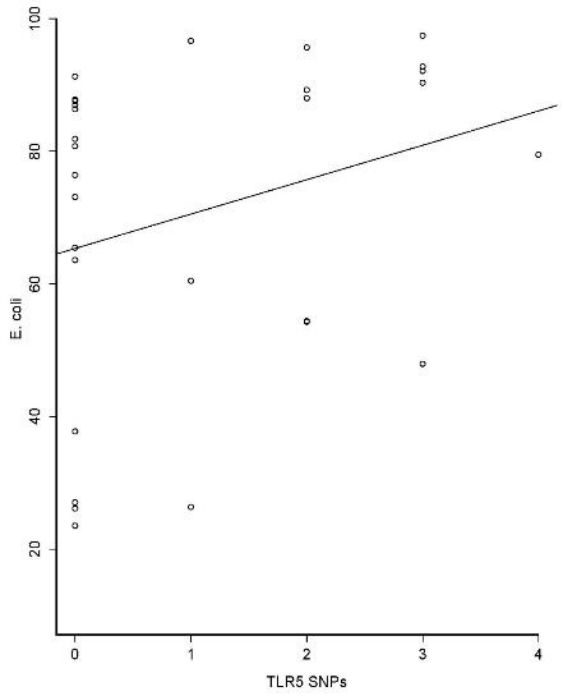

Figure D7. Plot comparing the number of nucleotide SNPs at TLR5 associated with *E. coli* bacteria killing capacity in red wolves (*Canis rufus*) and sympatric coyotes (*Canis latrans*). Higher killing capacity was significantly correlated with more TLR5 SNPs in mixed models controlling for year and family group (95% CI 7.45-23.87).

APPENDIX E: SUPPLEMENTAL FIGURES AND TABLES FROM CHAPTER 6

Table E1. Primers and primer conditions used to amplify major histocompatibility complex genes in red wolves (*Canis rufus*) and coyotes (*Canis latrans*).

Gene Exon	Sequence ('5-'3)	PCR conditions	Citation
DRB exon 2 (F)	CCGTCCCCACAGCACATTTC	95°Cx4',40x(95°Cx30",57°Cx 60",72°Cx60"), 72°Cx10'	Wagner et al. 1996, Wagner et al. 1998, Hedrick et al. 2002, Kennedy et al. 2007
DRB exon 2 (R)	TGTGTCACACACCTCAGCACCA		Wagner et al. 1996, Wagner et al. 1998, Hedrick et al. 2002, Seddon and Ellegren 2002
DQA exon 2 (F)	CTCAGCTGACCATGTTGC	95°Cx4',40x(95°Cx30",62°Cx 60",72°Cx60"), 72°Cx10'	Kennedy et al. 2004, Wilbe et al. 2009
DQA exon 2 (R)	GGACAGATTCAGTGAAGAGA		Wagner et al. 1996, Seddon and Ellegren 2002, Kennedy et al. 2007, Wilbe et al. 2009
DQB exon 2 (F)	TCACTGGCCCGGCTGTCTCC	95°Cx4',15x(95°Cx40",73°-0.5°C each cycle x 60",72°Cx60"),	Wagner et al. 1998, Seddon and Ellegren 2002
DQB exon 2 (R)	GGTGCGCTCACCTCGCCGCT	25x(95°Cx40",66°x60",72°Cx60"), 72°Cx10'	Wagner et al. 1998, Seddon and Ellegren 2002

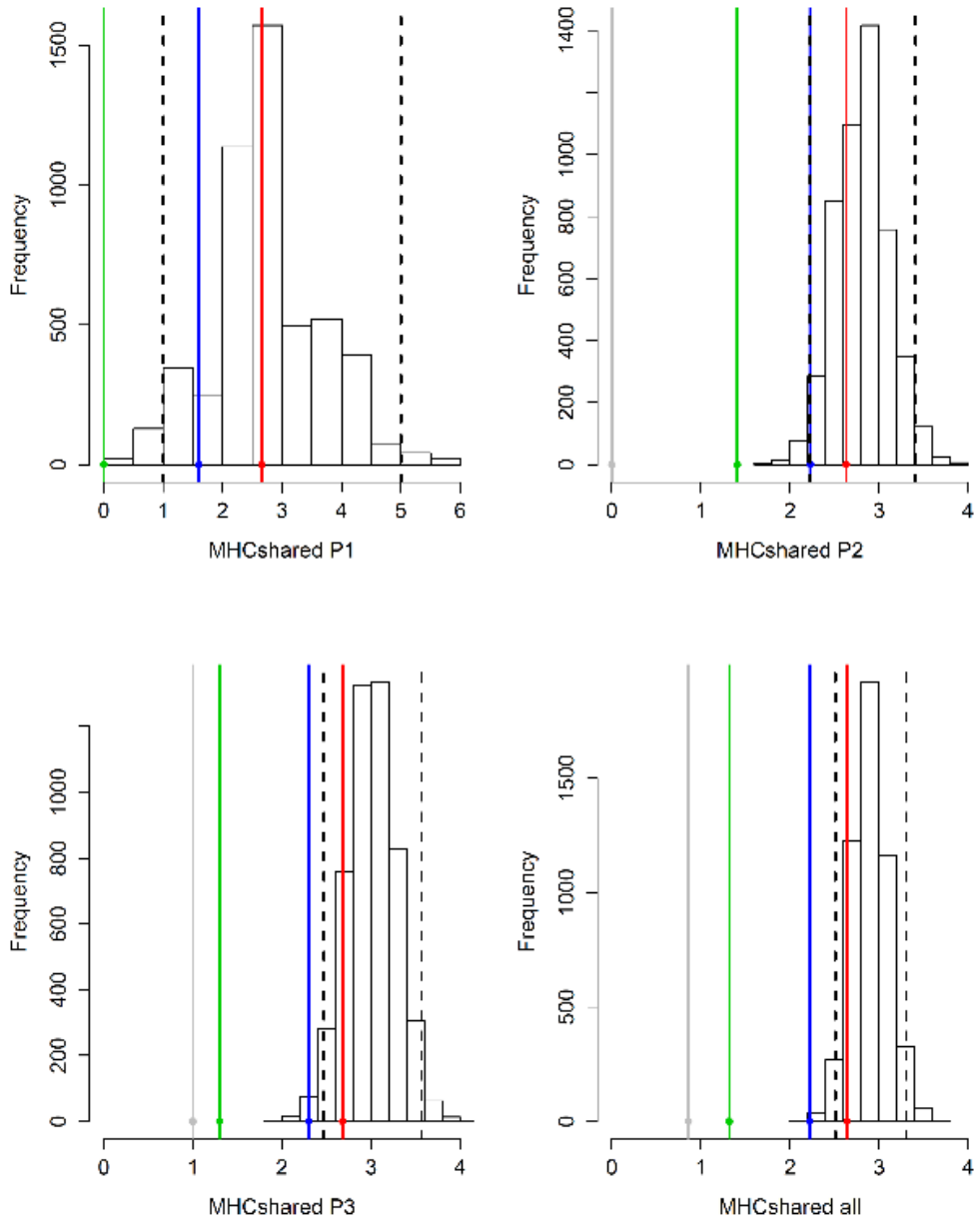

Figure E1. Mean number of shared MHC class II genes DRB1, DQB1, and DQA1 alleles of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random pairings of red wolf pairs that successfully reproduced. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

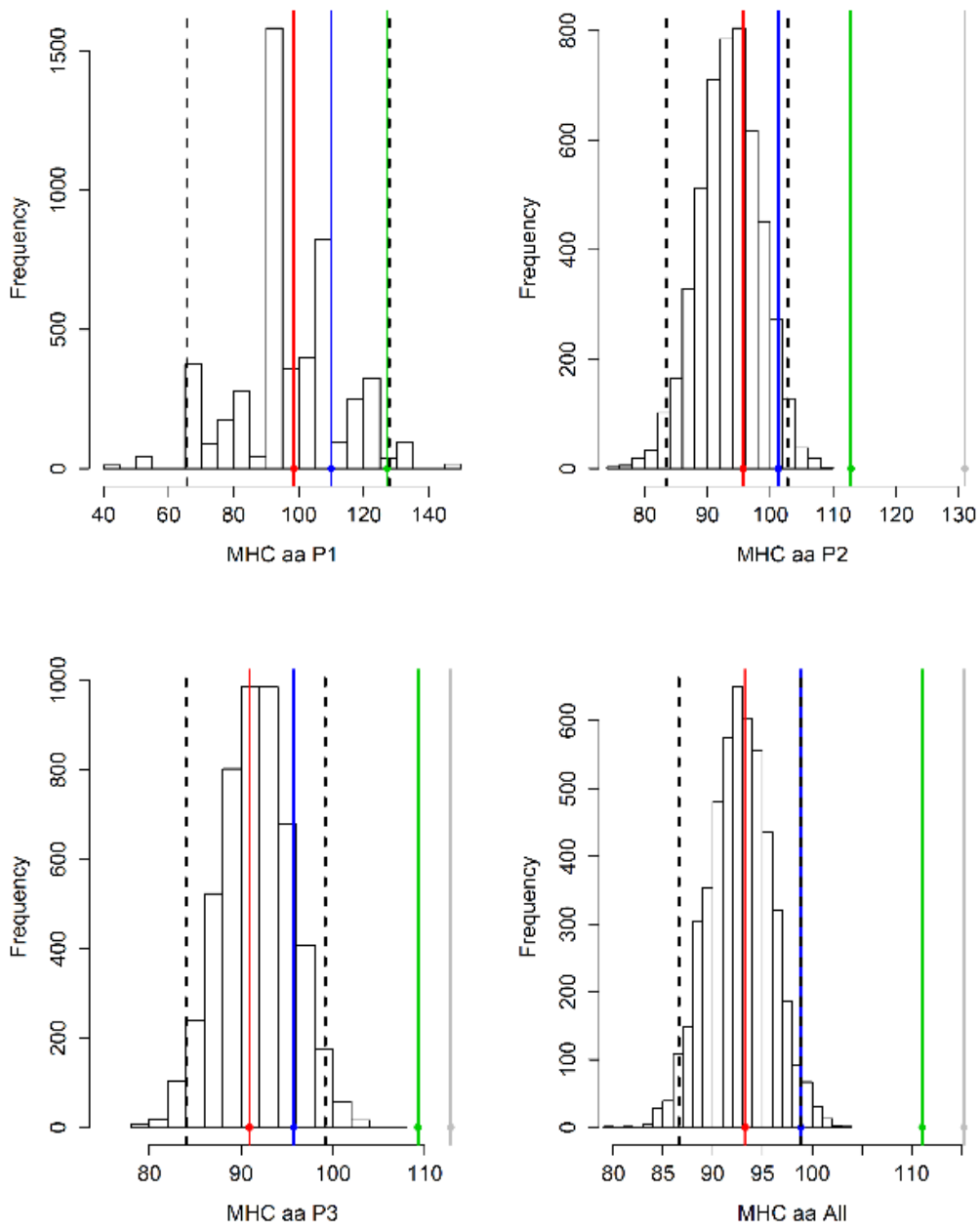

Figure E2. Mean combined MHC class II genes DRB1, DQB1, and DQA1 amino acid distance of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random pairings of red wolf pairs that successfully reproduced. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

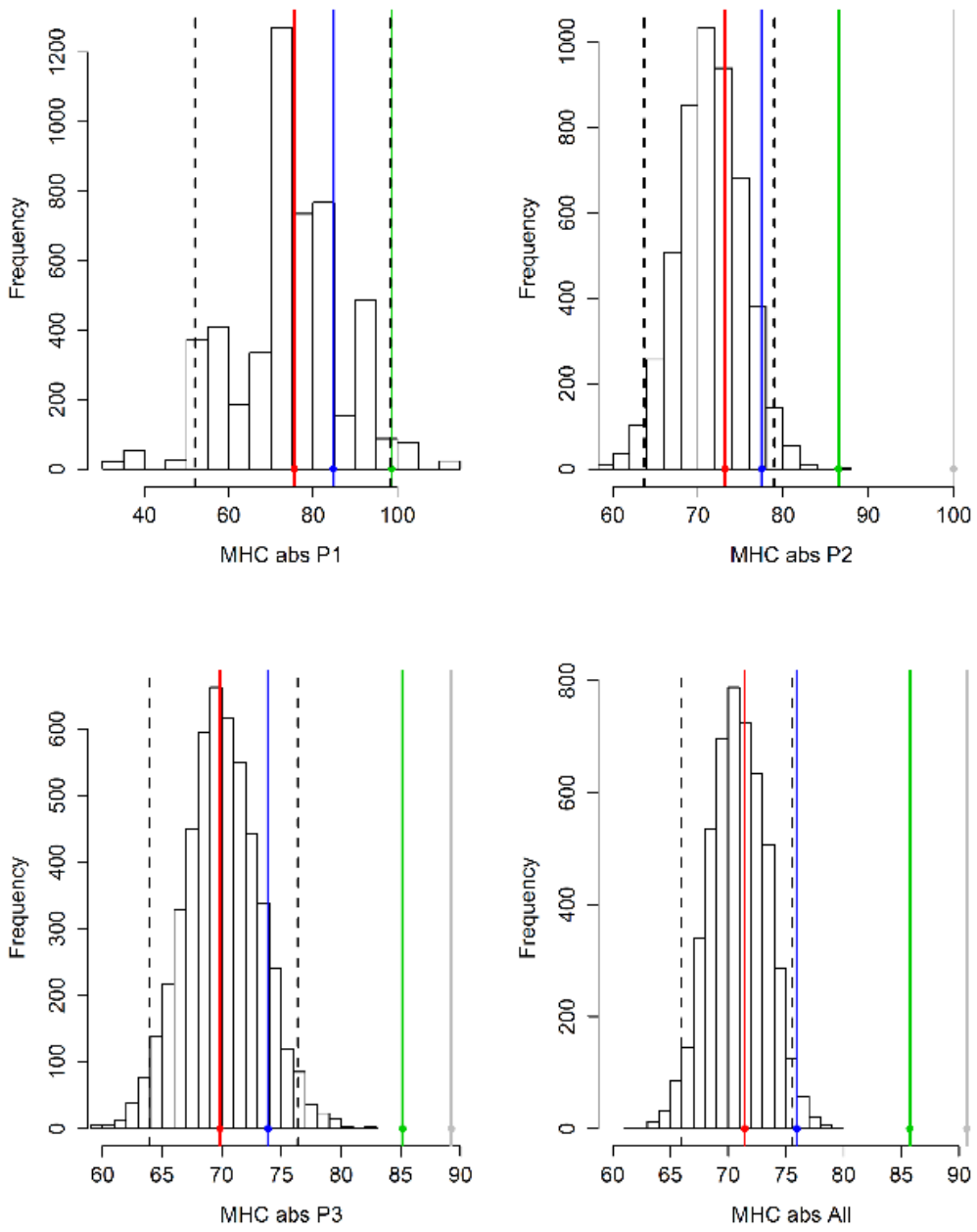

Figure E3. Mean combined MHC class II genes DRB1, DQB1, and DQA1 amino acid distance at antigen-binding sites (ABS) of observed red wolf pairs (red line), red wolf-hybrid and red wolf-coyote pairs (green line), red wolf-coyote pairs (gray line), and all pairs (blue line), compared to random expectations. The frequency distribution (histogram) shows mean values generated from 5000 simulations of random pairings of red wolf pairs that successfully reproduced. 95% confidence intervals (dashed line) indicate cut-offs for significant deviations from random mating. Simulations were conducted separately base on management priorities; Phase 1 (P1: 1996-1998), Phase 2 (P2: 1999-2005), Phase 3 (P3: 2006-2013), and for all time periods combined (All: 1996-2013).

VITA

Kristin Elise Brzeski was born in Milwaukee, WI. She attended Loyola University of Chicago from 2001-2005, where she pursued a Bachelor of Science degree in Environmental Science and competed as a Division I Track & Field athlete. During Kristin's bachelor's work, she spent a summer doing forest carnivore surveys in the Sierra Nevada mountain range, sparking her interest in wildlife biology. After completing her undergraduate degree, Kristin worked for the United States Department of Agriculture as a tree climber surveying for invasive pests before deciding to become more involved in wildlife conservation and management. Kristin sought a Master of Science in Wildlife Biology at Humboldt State University in 2007 under Dr. Micaela Szykman Gunther where she studied river otter ecology with noninvasive genetics. Kristin deepened her research focus in Conservation Genetics with her dissertation research evaluating the broad impacts of inbreeding on endangered red wolves with PhD adviser Dr. Sabrina Taylor in 2010. After spending several seasons collecting parasite data and learning about wild canid ecology, Kristin is scheduled to finish her PhD in 2015.